ISSN 1949-8454 (online)

# World Journal of *Biological Chemistry*

*World J Biol Chem* 2020 November 27; 11(3): 76-118

Published by Baishideng Publishing Group Inc

WJBC

World Journal of Biological Chemistry

Contents

Irregular Volume 11 Number 3 November 27, 2020

*World Journal of Biological Chemistry*

**Irregular Volume 11 Number 3 November 27, 2020**

## Contents




**NAME OF JOURNAL**
*World Journal of Biological Chemistry*

**ISSN**
ISSN 1949-8454 (online)

**LAUNCH DATE**
July 26, 2010

**FREQUENCY**
Irregular

**EDITORS-IN-CHIEF**
Vsevolod Gurevich, Chun-Peng Wan

**EDITORIAL BOARD MEMBERS**
https://www.wjgnet.com/1949-8454/editorialboard.htm

**PUBLICATION DATE**
November 27, 2020





**INSTRUCTIONS TO AUTHORS**
https://www.wjgnet.com/bpg/gerinfo/204

**GUIDELINES FOR ETHICS DOCUMENTS**
https://www.wjgnet.com/bpg/GerInfo/287

**GUIDELINES FOR NON-NATIVE SPEAKERS OF ENGLISH**
https://www.wjgnet.com/bpg/gerinfo/240

**PUBLICATION ETHICS**
https://www.wjgnet.com/bpg/GerInfo/288

**PUBLICATION MISCONDUCT**
https://www.wjgnet.com/bpg/gerinfo/208

**ARTICLE PROCESSING CHARGE**
https://www.wjgnet.com/bpg/gerinfo/242

**STEPS FOR SUBMITTING MANUSCRIPTS**
https://www.wjgnet.com/bpg/GerInfo/239

**ONLINE SUBMISSION**
https://www.f6publishing.com




E-mail: bpgoffice@wjgnet.com https://www.wjgnet.com

World Journal of Biological Chemistry

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

*World J Biol Chem* 2020 November 27; 11(3): 76-98

DOI: 10.4331/wjbc.v11.i3.76

ISSN 1949-8454 (online)

REVIEW

# Current understanding of glucose transporter 4 expression and functional mechanisms

Tiannan Wang, Jing Wang, Xinge Hu, Xian-Ju Huang, Guo-Xun Chen

**ORCID number:** Tiannan Wang 0000-0001-5584-3628; Jing Wang 0000-0003-3663-8332; Xinge Hu 0000-0002-3253-7537; Xian-Ju Huang 0000-0001-8130-1961; Guo-Xun Chen 0000-0001-6226-4050.

**Author contributions:** Wang T, Wang J and Hu X conducted the PubMed Search; Wang T, Wang J, Hu X, Huang XJ and Chen GX outlined and wrote the manuscript.

**Conflict-of-interest statement:** All authors declare that there is no conflict of interest to report.



**Manuscript source:** Invited manuscript

**Specialty type:** Biochemistry and molecular biology

**Tiannan Wang, Xinge Hu, Guo-Xun Chen,** Department of Nutrition, The University of Tennessee, Knoxville, TN 37996, United States

**Jing Wang, Xian-Ju Huang,** College of Pharmacy, South-Central University for Nationalities, Wuhan 430074, Hubei Province, China

**Corresponding author:** Guo-Xun Chen, PhD, Associate Professor, Department of Nutrition, The University of Tennessee, Room 229 Jessie Harris Building, 1215 West Cumberland Avenue Knoxville, TN 37996, United States. gchen6@utk.edu

## Abstract

Glucose is used aerobically and anaerobically to generate energy for cells. Glucose transporters (GLUTs) are transmembrane proteins that transport glucose across the cell membrane. Insulin promotes glucose utilization in part through promoting glucose entry into the skeletal and adipose tissues. This has been thought to be achieved through insulin-induced GLUT4 translocation from intracellular compartments to the cell membrane, which increases the overall rate of glucose flux into a cell. The insulin-induced GLUT4 translocation has been investigated extensively. Recently, significant progress has been made in our understanding of GLUT4 expression and translocation. Here, we summarized the methods and reagents used to determine the expression levels of *Slc2a4* mRNA and GLUT4 protein, and GLUT4 translocation in the skeletal muscle, adipose tissues, heart and brain. Overall, a variety of methods such real-time polymerase chain reaction, immunohistochemistry, fluorescence microscopy, fusion proteins, stable cell line and transgenic animals have been used to answer particular questions related to GLUT4 system and insulin action. It seems that insulin-induced GLUT4 translocation can be observed in the heart and brain in addition to the skeletal muscle and adipocytes. Hormones other than insulin can induce GLUT4 translocation. Clearly, more studies of GLUT4 are warranted in the future to advance of our understanding of glucose homeostasis.

**Key Words:** Glucose transporter 4; Insulin; Skeletal muscle; Adipocytes; Brain; Heart; Antibodies

**Country/Territory of origin:** United States

**Peer-review report's scientific quality classification**
Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C, C
Grade D (Fair): D
Grade E (Poor): 0

**Received:** June 26, 2020
**Peer-review started:** June 26, 2020
**First decision:** August 9, 2020
**Revised:** August 22, 2020
**Accepted:** September 22, 2020
**Article in press:** September 22, 2020
**Published online:** November 27, 2020

**P-Reviewer:** Merigo F, Salceda R, Soriano-Ursúa MA
**S-Editor:** Ma YJ
**L-Editor:** A
**P-Editor:** Ma YJ

**Core Tip:** Glucose transporter 4 (GLUT4) can be detected not only in the skeletal muscle and adipocytes, but also in the brain and heart. In addition to the translocation from vesicles in the cytosol to the cell membrane by insulin, the expression levels of Slc2a4 mRNA and GLUT4 proteins are also regulated by many factors. A variety of methods and antibodies from various sources have been used to evaluate GLUT4 expression and translocation.

**Citation:** Wang T, Wang J, Hu X, Huang XJ, Chen GX. Current understanding of glucose transporter 4 expression and functional mechanisms. *World J Biol Chem* 2020; 11(3): 76-98
**URL:** https://www.wjgnet.com/1949-8454/full/v11/i3/76.htm
**DOI:** https://dx.doi.org/10.4331/wjbc.v11.i3.76

## INTRODUCTION

Currently, diabetes is a problem of public health[1]. Based on the American Diabetes Association definition, diabetes is a serious chronic health condition of your body that causes blood glucose levels to rise higher than normal, which will lead to multiple complications if hyperglycemia is left untreated or mismanaged[2]. Diabetes occurs when your body cannot make insulin or cannot effectively respond to insulin to regulate blood glucose level. There are two type of diabetes, insulin-dependent type 1 diabetes mellitus (T1DM) and -independent type 2 diabetes mellitus (T2DM). T2DM accounts for about 90% to 95% of all diagnosed cases of diabetes, and is due to the lack of responses to insulin in the body[3]. Insulin resistance is a characteristic of T2DM. For a person with diabetes, a major challenge is to control or manage blood glucose level. Glucose is a common molecule used for production of energy or other metabolites in cells. As a quick energy source, glucose can be metabolized aerobically or anaerobically depending on the availability of oxygen or cell characteristics[4]. Glucose is a hydrophilic molecule, and cannot diffuse into or out of a cell freely. It needs transporters to cross the cell membrane. Glucose transporters (GLUTs) are proteins that serve this purpose.

GLUTs are members of the major facilitator superfamily (MFS) transporters, which are responsible for the transfer of a large array of small molecules such as nutrients, metabolites and toxins across the cell membrane[5]. Multiple members have been identified in each family of MFS transporters, and changes of their functions have been associated with a number of diseases[5]. Members of MFS transporters have 12 transmembrane helices, and transport their substrates as uniporters, symporters or antiporters[5]. Upon binding of the substrates on side of the membrane, a conformation change occurs, which is achieved through coordinative interactions of those helices through a "clamp-and-switch" mechanism. Structural studies have shown that the substrate specificity is achieved through the conserved amino acid residues within each family[5]. Thus, it is important to understand GLUT functions, expressions and regulations for the control of blood glucose homeostasis.

### *Insulin and GLUTs*

Dietary starch is first digested into glucose before being absorbed into the body and utilized[4]. The first transporter identified is GLUT1, which is expressed universally in all cells, and responsible for basal glucose transport[6]. Insulin stimulates glucose utilization in the body. This is in part through the insulin-induced glucose uptake in the muscle and adipose tissues. In addition to insulin stimulation, physical activity can also increase glucose entering into the skeletal muscle cells[7]. The observation that insulin promotes the redistribution of GLUTs from intracellular locations to the plasma membrane in adipocytes began in the 1980s[8-10]. Few years later, the insulin-induced glucose transport was also found in muscle cells[11,12]. To understand the underlying mechanism of insulin-stimulated glucose uptake, antibodies against membrane glucose transport proteins were created[13]. Subcellular fractionation, cytokinin B (glucose-sensitive ligand), and glucose absorption into isolated vesicles were used to study the phenomenon. It was proposed that these GLUTs are moved from intracellular components to the plasma membrane of adipocytes and muscle cells upon insulin stimulation[6]. In 1988, a specific antibody against a GLUT sample preparation was created, which eventually led to the identification of a molecular

clone that encodes an insulin-induced GLUT from mouse adipocytes[6]. It was named GLUT4. Since the 1990s, fluorescent-labeled fusion proteins, GLUT4-specific antibodies, photoaffinity labeling reagents, immunofluorescence microscopy, and high-resolution electron microscope have been used to confirm the insulin-induced translocation and underlying mechanisms[6]

It has been widely accepted that insulin mainly stimulates transfer of GLUT4 from intracellular storage vesicles to the plasma membrane. Insulin stimulation accelerates the movement rate of GLUT4 containing vesicles to the cell membrane[14]. When more GLUT4 is on the plasma membrane, more glucose enters the cells without any change of the GLUT4 specific activity. During insulin stimulation, GLUT4 is not statically maintained in the plasma membrane but continuously recycled[6]. After insulin is removed, the amount of GLUT4 on the plasma membrane drops and the rate of movement returns to basal level.

Since identification and cloning of GLUT1, 13 additional GLUTs have been cloned using recombinant DNA techniques[15]. Based on their phylogeny or genetic and structural similarities, GLUTs are classified into three classes. Class I includes GLUTs 1-4, and GLUT14 which are responsible for glucose transfer. Class II consists of GLTUs 5, 7, 9 and GLUT 11 which are considered as fructose transporters. Class III contains GLTUs 6, 8, 10, 12 and GLUT 13[16]. All GLUTs have nearly 500 amino acid residues that form 12 transmembrane helices[15].

Each GLUT has its own unique affinity and specificity for its substrate, tissue distribution, intracellular location, regulatory mechanisms and physiological functions[17]. The most well studied and known members are GLUTs 1-6. GLUT1 is found evenly distributed in the fetal tissues. In human adults, GLUT1 Level is high in erythrocytes and endothelial cells. It is responsible for basal glucose uptake[18]. GLUT2 is expressed in the liver and pancreas, and contributes to glucose sensing and homeostasis[17]. In enterocytes, GLUT2 is responsible to transport the absorbed glucose, fructose and galactose out of the basolateral membrane to enter into the blood circulation through the portal vein[19]. GLUT3 just like GLUT1 is expressed in almost all mammalian cells and is responsible for the basal uptake of glucose. GLUT3 is considered as the main GLUT isoform expressed in neurons and the placenta, but has also been detected in the testis, placenta, and skeletal muscle[20-22]. GLUT5 is specific for uptake of fructose in a passive diffusion manner, and is expressed in the small intestine, testes and kidney[17]. GLUT6 is expressed in the spleen, brain, and leukocytes as well as in muscle and adipose tissue[15,23]. GLUT6 has been shown to move from the intracellular locations and plasma membrane of rat adipocytes in a dynamin-dependent manner[23]. Table 1[24-57] summarizes names, numbers of amino acids, Kms, expression profiles and potential functions of those GLUTs.

### *GLUT4 gene, its tissue distribution, and physiological functions*

Human GLUT4 has 509 amino acid residues and is encoded by *SLC2A4* gene in the human genome. It is mainly expressed in adipocytes and skeletal muscle. The unique N-terminal and COOH terminal sequences are responsible for GLUT4's response to insulin signaling and membrane transport[58]. The Km of GLUT4 is about 5 mmol/L. In response to insulin stimulation, intracellular vesicles containing GLUT4 are moved from cytosol to the cell membrane. As shown in Figure 1, insulin receptor is a tetramer with two alpha-subunits and two beta-subunits linked by disulfide bonds[59]. When insulin binds to its receptor on the cell membrane, insulin receptor beta subunits that contain tyrosine kinase domain autophosphorylate each other. The phosphorylated β-subunits recruit insulin receptor substrates (IRS) and phosphorylate them. Then phosphorylated IRSs bind to and activate phosphatidylinositol 3-kinase (PI3K) which is recruited to the plasma membrane and converts PIP2 to PIP3. On the plasma membrane, PI3K activates PIP3 dependent protein kinase, which phosphorylates and activates AKT (also referred to as protein kinase B, PKB). Akt activation triggers vesicle fusion, which results in the translocation of GLUT4 containing vesicles from intracellular compartments to the plasma membrane. The elevation of GLUT4 on the membrane leads to increase of glucose entry into the cell.

Upon refeeding, elevated glucose levels in the blood stimulates insulin secretion from pancreatic beta cells. Insulin stimulates GLUT4 translocation to the cell membrane, which increases glucose uptake in cells, and achieves glucose homeostasis[60,61]. After the insulin stimulation disappears, GLUT4 is transferred back into the cytosol from the plasma membrane. More than 90% of GLUT4 is located in the intracellular body, trans-Golgi network, and heterogeneous tube-like vesicle structure, *etc.*, which constitute the GLUT4 storage vesicle (GSV). In an unstimulated state, most GLUT4 is in the intracellular vesicles of muscle and adipocytes[62].

The amount of GLUT4 on the cell membrane is determined by the rate of the

**Table 1 Summary of glucose transporter family members**

| Protein (gene) | Amino acids | Km (mm) | Expression sites | Function/substrates | Ref. |
|---|---|---|---|---|---|
| GLUT1 (*SLC2A1*) | 492 | 3-7 | Ubiquitous distribution in tissues and culture cells | Basal glucose uptake; glucose, galactose, glucosamine, mannose | [24-30] |
| GLUT2 (*SLC2A2*) | 524 | 17 | Liver, pancreas, brain, kidney, small intestine | High-capacity low-affinity transport; glucose, galactose, fructose, glucosamine, mannose | [25-27,29-34] |
| GLUT3 (*SLC2A3*) | 496 | 1.4 | Brain and nerves cells | Neuronal transport; glucose, galactose, mannose | [25-27,29,30, 33-35] |
| GLUT4 (*SLC2A4*) | 509 | 5 | Muscle, fat, heart, hippocampal neurons | Insulin-regulated transport in muscle and fat; glucose, glucosamine | [25-27,29,31, 36,37] |
| GLUT5 (*SLC2A5*) | 501 | 6 | Intestine, kidney, testis, brain | Fructose | [25-27,29,30, 34,38-42] |
| GLUT6 (*SLC2A6*) | 507 | 5 | Spleen, leukocytes, brain | Glucose | [25-27,29,30, 43] |
| GLUT7 (*SLC2A7*) | 524 | 0.3 | Small intestine, colon, testis, liver | Fructose and glucose | [25-27,29,30, 38] |
| GLUT8 (*SLC2A8*) | 477 | 2 | Testis, blastocyst, brain, muscle, adipocytes | Insulin-responsive transport in blastocyst; glucose, fructose, galactose | [25-27,29,30, 44,45] |
| GLUT9 (*SLC2A9*) | Major 540, Minor 512 | 0.9 | Liver, kidney | Glucose, fructose | [25-27,29,30, 46-48] |
| GLUT10 (*SLC2A10*) | 541 | 0.3 | Heart, lung, brain, skeletal muscle, placenta, liver, pancreas | Glucose and galactose | [25-27,29,30, 48,49] |
| GLUT11 (*SLC2A11*) | 496 | 0.2 | Heart, muscle, adipose tissue, pancreas | Muscle-specific; fructose and glucose transporter | [25-27,29,30, 50-54] |
| GLUT12 (*SLC2A12*) | 617 | 4-5 | Heart, prostate, skeletal muscle, fat, mammary gland | Glucose | [25-27,29,30, 53,55] |
| GLUT13 (*SLC2A13*) | Rat 618, human 629 | 0.1 | Brain (neurons intracellular vesicles) | H+-myo-inositol transporter | [25-27,29,30, 56] |
| GLUT14 (*SLC2A14*) | Short 497, Long 520 | unknown | Testis | Glucose transport | [25-27,29,30, 57] |

movement from intracellular GSV to the cell membrane. In adipocytes and skeletal muscle cells, insulin increases the rate of GLUT4 translocation from GSVs to membrane and decreases the rate of GLUT4 movement from membrane back to the vesicles, which lead to elevation of GLUT4 content on the cell membrane by 2-3 times[63]. Moreover, in adipocytes, insulin increases the GLUT4 recirculation to maintain a stable and releasable vesicle[64].

So far, insulin-induced GLUT4 translocation has been studied extensively. However, questions still remain. Methods and reagents used to determine the expression levels of GLUT4 and its translocation mechanism deserve to be summarized and analyzed. Therefore, we searched the relevant articles in PubMed and investigated the methods and reagents used in the studies. "Glucose transporter 4" and "GLUT4" as the protein and "*SLC2A4*" as the gene name were used as keywords in the search. In order to have a more clearly overview, we further divided and focused on the search into three parts, GLUT4 in the skeletal muscle, GLUT4 in adipose tissues, and GLUT4 in heart and brain.

## GLUT4 IN THE SKELETAL MUSCLE

The term "muscle" covers a variety of cell types. Mammals have four main types of muscle cells: skeletal, heart, smooth, and myoepithelial cells. They are different in function, structure and development[65]. The skeletal muscle mass accounts for 40% of the total body mass, and the regulation of skeletal muscle glucose metabolism will significantly affect the body's glucose homeostasis[66,67]. Skeletal muscle is composed of many muscle fibers connected by collagen and reticular fibers. Each skeletal muscle fiber is a syncytium that derives from the fusion of many myoblasts. Myoblasts proliferate in large quantities, but once fused, they no longer divide. The fusion

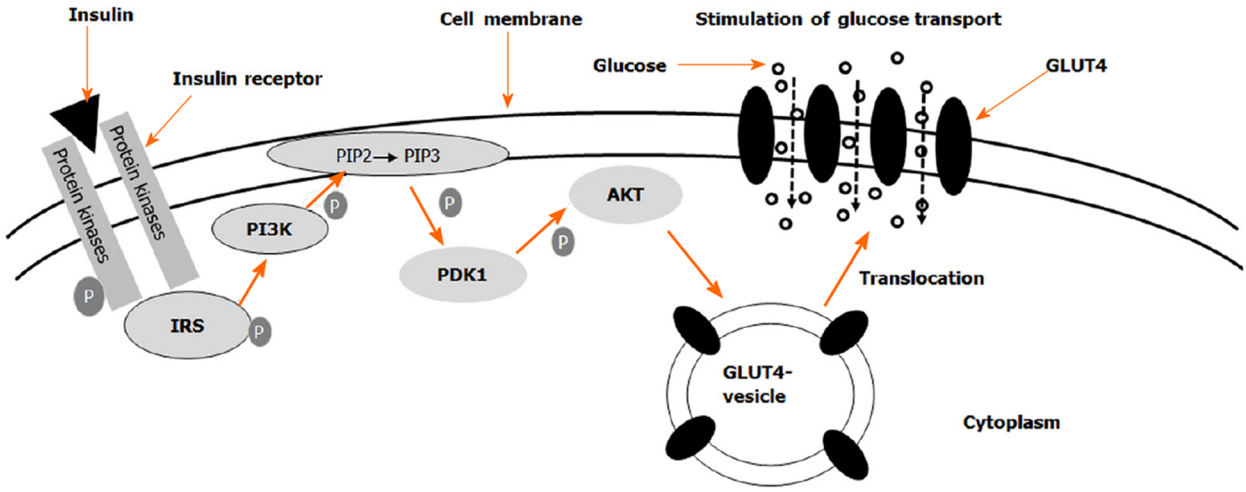


**Figure 1 Schematic of insulin-induced translocation of glucose transporter 4 from cytosol to the cell membrane.** The binding of insulin to its receptors initiates a signal transduction cascade, which results in the activation of Akt. Akt acts on the glucose transporter 4 (GLUT4) containing vesicles in the cytosol to facilitate their fusion with the cell membrane. When more GLUT4 molecules are present in the membrane, the rate of glucose uptake is elevated. GLUT4: Glucose transporter 4.

usually follows the onset of myoblast differentiation[65]. Different fiber types have distinct contractile and metabolic properties[68]. The skeletal muscle maintains skeletal structure and essential daily activities[69]. Also, it is a source of proteins that can be broken down into amino acids for the body to use.

Insulin stimulates glucose uptake and utilization in the skeletal muscle. GLUT4 plays a key role in the uptake process. Glucose can be stored as glycogen, which is used as a quick source of energy in physical activity[70]. In the skeletal muscle, exercise helps increase insulin sensitivity and stimulates *SLC2A4* gene transcription[60]. Physiological factors such as the type of muscle fibers can also affect the GLUT4 Level. An increase in physical activity will induce the GLUT4 Levels, whereas a decrease in activity level will reduce GLUT4[68]. The skeletal muscle not only maintains the activities, but also regulates the glucose homeostasis in the body, which plays a key role in the development of metabolic diseases[69]. Obesity and T2DM have a negative impact on skeletal muscle glucose metabolism[71].

To review the methods and reagents of GLUT4 studies in skeletal muscle, "GLUT4, skeletal muscle" and "SLC2A4, skeletal muscle" as keywords were used to search the PubMed database to retrieve relevant articles. The skeletal muscle is a highly specialized tissue made of well-organized muscle fibers. The unique structural characteristic of muscle inherits difficulties to be lysed for biochemical studies. Therefore, we want to focus on the sample preparation of skeletal muscle in GLUT4 studies. The retrieved articles were screened mainly according to the research methods and reagents used for skeletal muscle preparation, experimental groups included, *Slc2a4* mRNA and GLUT4 protein measurements and the source of GLUT4 antibodies obtained. In the end, 10 representative articles were selected for analysis and summary as shown in Table 2[72-81].

Overall, the current research methods of GLUT 4 studies in skeletal muscle are listed below: (1) Samples were homogenized to prepare membrane fractions for analysis of GLUT 4 in western blot using monoclonal or polyclonal antibodies; (2) Real-time polymerase chain reaction (PCR) was used to determine the mRNA abundance of *Slc2a4*; (3) Immunocytochemical staining was used to detect GLUT4 *in situ*. The fibers were labeled for GLUT4 by a preembedding technique and observed as whole mounts by immunofluorescence microscopy or after sectioning, by immunogold electron microscopy. Preembedding is a technique to label GLUT4 immediately after tissues or cells are collected, which allows that the antibody interacts with the antigen before denaturation; (4) Muscle cell lines stably expressing tagged GLUT4 were established to study the translocation; and (5) Radiolabeled 2-deoxyglucose was used to determine the glucose uptake in muscle tissue slices.

The antibodies used in these articles were from Santa Cruz, Millipore, East Acres, Biogenesis and other sources not specified. Only two of the publications have a positive control group of GLUT4 expression using overexpression of a fusion protein and tissue preparation as a standard for determination. Positive controls are important

**Table 2 Recent studies of glucose transporter 4 expression and translocation in the skeletal muscle**

| Methods | Materials | Comparisons | Observations/conclusions | Ref. |
|---|---|---|---|---|
| Western blot. | Cell fractions of rat L6 myotubes, 3T3-L1, and mouse muscle and adipose tissues. Anti-GLUT4 from Santa Cruz Biotechnology (1:1000). | Cell: Total cell lysate *vs* membrane fractions. Mouse tissues: Control *vs* high-fat diets. | Insulin treatments increases GLUT4 levels in membrane fractions without any change in the total cell lysate. GLUT4 levels in adipose tissue and muscle of mice fed a high-fat diet are lower in all fractions than that fed the control diet. | [72] |
| Western blot. | Whole cell and cell fractions from rat L6 and mouse C2C12 muscle cells, and soleus muscle of hind limb from mice. Anti-GLUT4 from Santa Cruz. Biotechnology (1:1000). | Whole cell lysate *vs* membrane fractions. Treatments without or with insulin or AICAR. | GLUT4 translocation occurs in L6 myotubes and 3T3-L1 adipocytes stimulated by insulin and AICAR. GLUT4 translocation occurs in muscle at 15 to 30 minutes and in adipose tissue at 15 minutes after glucose treatment. | [73] |
| Western blot. | Giant sarcolemmal vesicles from soleus muscles of Sprague-Dawley rats. Anti-GLUT4 from Millipore (1:4000). | Tissue samples without or with insulin released in the presence of glucose as a stimulant and lipid as a control. | A glucose-dependent insulinotropic polypeptide increases glucose transport and plasma membrane GLUT4 protein content. | [74] |
| Real-time PCR for *Slc2a4* mRNA levels. | Total RNA of the skeletal muscle from male C57BL/6J and ICR mice fed different diets. | mRNA levels in muscle samples from mice fed the control or CLA supplement diet | Dietary CLA does not affect *Slc2a4 mRNA* levels in the mouse skeletal muscle | [75] |
| Western blot. | Preparations of sarcolemmal membrane fractions and crude lysates from male Muscovy ducklings. Anti-GLUT4 from East Acres (1:500). | GLUT4 from a unique crude membrane fraction of rat skeletal muscle was used as an arbitrary unit and from erythrocyte ghost as a negative control | Polyclonal antibodies detect a protein of similar size (approximately 45 kDa) of GLUT4 in the crude membrane preparations from rat (positive control) and duckling skeletal muscle. No signal was obtained for rat erythrocyte ghost membrane preparation. | [76] |
| ATB-BMPA-labelling of glucose transporters, Immunoprecipitation, liquid-scintillation counting, Western blot. | Tissue samples of isolated and perfused EDL or soleus muscle from GLUT1 transgenic C57BL′KsJ-Leprdbj and control mice. Anti-GLUT4 (R1184; C-terminal) from an unknown source. | Non-transgenic mice *vs* transgenic mice. | Basal levels of cell-surface GLUT4 in isolated or perfused EDL are similar in transgenic and non- transgenic mice. Insulin induces cell-surface GLUT4 by 2-fold in isolated EDL and by 6-fold in perfused EDL of both transgenic and non-transgenic mice. Western blot results were not shown. | [77] |
| Preembedding technique (immune reaction occurs prior to resin embedding to label GLUT4), and observations of whole mounts by immunofluorescence microscopy, or after sectioning by immunogold electron microscopy. | Muscle samples from male Wistar rats. Anti-GLUT4 (C-terminal, 1:1000), and anti-GLUT4 (13 N-terminal, 1:500) from unknown species. | Rats were divided in four groups: Control, contraction received saline, insulin and insulin plus contraction groups. They received glucose followed by insulin injection. | Two populations of intracellular GLUT4 vesicles are differentially recruited by insulin and muscle contractions. The increase in glucose transport by insulin and contractions in the skeletal muscle is due to an additive translocation to both the plasma membrane and T tubules. Unmasking of GLUT4 COOH-terminal epitopes and changes in T tubule diameters does not contribute to the increase in glucose transport. | [78] |
| Immunoprecipitation, and Western blot. | Membrane fractions from skeletal muscle of male Wistar rats treated without or with insulin. Anti-GLUT-4 from Genzyme, Anti-GLUT-4 from Santa Cruz Biotechnology. | Crude membrane preparations and cytosolic fractions in samples of rats treated without or with insulin. | In vitro activation of PLD in crude membranes results in movement of GLUT4 to vesicles/microsomes. This GLUT4 translocation is blocked by the PLD inhibitor, neomycin, which also reduces insulin-stimulated glucose transport in rat soleus muscle. | [79] |
| Western blot for GLUT4 protein in homogenates of epitrochlearis muscles. Tissue slices labeled with 2-[1,2-$^3$H]-deoxy-d-glucose and counted in a gamma counter. | Muscle homogenate and slices from male Sprague-Dawley rats. Anti- GLUT4 from Dr. Osamu Ezaki. | Sedentary control *vs* a 5-day swimming training group. | The change of insulin responsiveness after detraining is directly related to muscle GLUT-4 protein content. The greater the increase in GLUT-4 protein content induced by training, the longer an effect on insulin responsiveness lasts after training. | [80] |
| Immunofluorescence for membrane preparations, and 2-Deoxyglucose uptake in isolated skeletal | Membrane preparations from L6 cells over-expressing GLUT4myc. Isolated | L6 cells over-expressing GLUT4myc treated without or with Indinavir. | HIV-1 protease inhibitor indinavir at 100 μmol/l inhibits 80% of basal and insulin-stimulated 2-deoxyglucose uptake in L6 myotubes with stable expression of GLUT4myc. | [81] |

| | |
|---|---|
| muscles. | skeletal muscle samples from mice. Anti-GLUT4 from Biogenesis. |

AICAR: 5-aminoimidazole-4- carboxamide ribonucleotide; CLA: Conjugated linoleic acid; EDL: Extensor digitorum longus; HIV: Human immunodeficiency virus; ICR: Institute of Cancer Research; PLD: Phospholipase D; GLUT4: Glucose transporter 4.

when Western blot and fusion protein immunofluorescence methods are used to determine the GLUT4 protein levels.

According to studies summarized in Table 2, the following key points can be obtained. Insulin and muscle contraction increase glucose uptake in the skeletal muscle, which is associated with increases of GLUT4 content and its translocation. Neomycin, a phospholipase D inhibitor, blocks GLUT4 translocation. In the skeletal muscle isolated from GLUT1 transgenic mice, insulin-induced GLUT4 translocation response is lost, which is not due to down-regulation of GLUT4 expression. Conjugated linoleic acid in the diet does not affect the *Slc2a4* mRNA expression in the skeletal muscle. Indinavir, an HIV-1 protease inhibitor, can block the glucose uptake mediated by GLUT4 in normal skeletal muscle and adipocytes without or with insulin stimulation. More studies are anticipated to elucidate how insulin resistance and T2DM affect the functions of GLUT4 system and whether overnutrition plays a role in it.

## GLUT4 IN ADIPOSE TISSUES

The ability for an organism to store excessive amount of energy in the form of fat is helpful for it to navigate a condition of an uncertain supply of food[82]. Adipocytes, the main type of cells in adipose tissue, are not only a place for fat storage, but also endocrine cells to secrete cytokines for the regulation of whole body energy homeostasis[82]. Based on the mitochondrial content and physiological functions, adipocytes are divided into white, beige and brown fat cells. Structurally, 90% of the cell volume of a white adipocyte is occupied by lipid droplets. In normal-weight adults, white adipose tissue accounts for 15% to 20% of body weight[83,84]. Excessive accumulation of body fat results in the development of obesity, which can lead to the development of T2DM if not managed[85,86]. In addition, adipose tissues secrete cytokines such as leptin and adiponectin with abilities to regulate food intake and insulin sensitivity[87]. GLUT4 is expressed in adipocytes, where insulin stimulates its translocation from intracellular locations to the cell membrane, which leads to increase of glucose uptake[88]. High expression levels of GLUT4 in adipose tissue can enhance insulin sensitivity and glucose tolerance[89].

Insulin-induced GLUT4 translocation in adipose tissue and skeletal muscle has been studied extensively. Overall, in recent years, great progress has been made in the

understanding of GLUT4 vesicles movement, the fusion of the vesicles with the cell membrane, and the translocation mechanism in response to insulin. As shown in Figure 2, in adipocytes of an adipose depot, GLUT4 vesicles move from the specialized intracellular compartment to the cell periphery (near cell membrane), which is followed by tethering and docking. Tethering is the interaction between GLUT4 vesicles and the plasma membrane. Docking is the assembly of the soluble N-ethylmaleimide-sensitive factor-attachment protein (SNAP) receptor (SNARE) complex. Fusion occurs when the lipid bilayers of the vesicles with GLUT4 and the cell membrane merge[90]. The actin cytoskeleton system plays an important role in retaining GLUT4 vesicles in adipocytes. After insulin stimulation, remodeling of cortical actin causes the release GLUT4 vesicles to the plasma membrane[90-92]. β-catenin plays an important role in regulating the transport of synaptic vesicles. The amount of GLUT4 within the insulin sensitive pool is determined by the β-catenin levels in adipocytes, which allows GLUT4 translocate to the cell membrane in response to insulin stimulation[93,94].

To summarize the current methods and reagents used for GLUT4 analysis in adipose tissues and adipocytes, "GLUT4, 3T3-L1", and "GLUT4, adipocytes" were used to search the literature published in the past 15 years. We ignored those studies that only measured *Slc4a2* mRNA, lacked the focus on adipocytes, and did not have full text versions. The resulted 30 articles were analyzed and summarized in Tables 3[95-112] and 4[113-124]. Table 3 contains 18 articles and summarizes the effects of drugs or bioactive compounds on *Slc2a4* mRNA and GLUT4 protein expressions, and GLUT4 translocation. Table 4 contains 12 articles and summarizes studies of the regulatory mechanisms of GLUT4 system.

There are 11 and 2 articles respectively using real-time PCR and Northern blot to evaluate *Slc2a4* mRNA levels. Western blot and ELISA are used in 24 articles to detect GLUT4 protein content. The antibodies were from Santa Cruz (6), Millipore (3), Cell Signaling Technology (4), Chemicon (4), Abcam (3), Pierce (1), Oxford (1) and Signalway (1). Two articles did not indicate the antibody sources. One article used antibodies from two companies. Nine articles indicated the dilutions of antibodies, and only 3 articles included the production catalog number. Immunofluorescence was used to detect the content and translocation of GLUT4 protein in 9 articles. Three articles used flow cytometry to detect GLUT4 protein. Twenty four of these 30 articles directly assessed levels of *Slc2a4* mRNA or GLUT4 protein. The remaining 6 of the 30 articles measured GLUT4 protein using fusion proteins. No article has a positive control group that uses overexpression of GLUT4 *via* a recombinant construct or purified recombinant GLUT4 protein.

As shown in Table 3, GLUT4 translocation, and *Slc2a4* mRNA and GLUT4 expression levels in 3T3-L1 cells can be regulated by bioactive compounds, crude extract of herbs, agonists of nuclear receptors, proteins and chemical drugs. *Sl2a4* mRNA or/and GLUT4 expressions in 3T3-L1 cells or adipose tissues can be increased by kaempferitrin, GW9662, inhibitor of p38 kinase, estradiol, crude extract of stevia leaf, fargesin, phillyrin, selenium-enriched exopolysaccharide, aspalathin-enriched green rooibos extract, bone morphogenetic proteins 2 and 6, and glucose pulse. On the other hand, *Slc2a4* mRNA and GLUT4 protein levels can be reduced by luteolin, and shilianhua extract in 3T3-L1 cells. GLUT4 translocation can be enhanced by kaempferitrin, curculigoside and ethyl acetate fractions, gallic acid, 6-hydroxydaidzein and ginsenoside Re, and reduced by green tea epigallocatechin gallate.

As shown in Table 4, a variety of methods have been used to study the regulatory mechanisms of GLUT4 system. 3T3-L1 cells have been the major model in those studies. In addition to the insulin, pathways involved in the *Slc2a4* gene expression, GLUT4 protein expression and its translocation include cannabinoid receptor 1 (CB1), ADP-ribosylation factor-related protein 1, MiR-29 family, proteasome system, estrogen pathway, oxidative stress *via* CCAAT/enhancer-binding protein alpha (C/EBPα), obesity development, differentially expressed in normal and neoplastic cells domain-containing protein 4C, nuclear factor-κB, Akt and Akt substrate of 160 kDa, phosphatidylinositol-3,4,5-trisphosphate dependent Rac exchange factor 1, secreted protein acidic and rich in cysteine (SPARC), sterol regulatory element-binding protein 1 (SREBP-1), and AMP-activated protein kinase (AMPK) pathway. CB1 receptor antagonists increase *Slc2a4* mRNA and GLUT4 protein expressions through NF-kB and SREBP-1 pathways. Akt pathway regulates the rate of vesicle tethering/fusion by controlling the concentration of primed, and fusion-competent GSVs with the plasma membrane. Inhibition of the SPARC expression reduces *Slc2a4* mRNA and GLUT4 expressions. The expressions of C/EBPα and δ alter the C/EBP-dimer formation at the *Slc2a4* gene promoter, which regulates its transcription. Inhibition of differentially expressed in normal and neoplastic cells domain-containing

**Table 3 Recent studies of effects of bioactive compounds and chemical drugs on glucose transporter 4 expression and translocation in adipocytes**

| Methods | Materials | Comparisons | Conclusions | Ref. |
|---|---|---|---|---|
| Immunoprecipitation, dual fluorescence immunostaining, Western blot. | 3T3-L1, anti-GLUT4 from Santa Cruz Biotechnology (1:200). | Treatments without or with kaempferitrin. | Kaempferitrin treatment upregulates total GLUT4 expression and its translocation in 3T3-L1 cells. | [95] |
| Subcellular fractionations, Western blot. | 3T3-L1, anti-GLUT4 from Cell Signaling Technology (1:1000). | Treatments without or with epigallocatechin gallate. | Green tea epigallocatechin gallate suppresses insulin-like growth factor-induced-glucose uptake *via* inhibition of GLUT4 translocation in 3T3-L1 cells. | [96] |
| Western blot. | 3T3-L1, anti-GLUT4 from Santa Cruz Biotechnology. | Treatments without or with GW9662. | GW9662 increases the expression of GLUT4 protein in 3T3-L1 cells. | [97] |
| Immunoprecipitation, Western blot. | 3T3-L1, anti-GLUT4 from Chemicon. | Treatments without or with p38 inhibition. | Inhibition of p38 enhances glucose uptake through the regulation of GLUT4 expressions in 3T3-L1 cells. | [98] |
| Western blots, Real-time PCR, Electrophoretic mobility shift assay, Immunofluorescence. | Adipose tissues of *Esr1* deletion and wild type female mice, 3T3-L1, anti-GLUT4 from Merck/Millipore for Western blot (1:4000), and for immunofluorescence (1:100). | Tissue and cells without or with gene deletion. | Estradiol stimulates adipocyte differentiation and *Slc2a4* mRNA and GLUT4 protein expressions in an ESR1/CEBPA mediated manner in *vitro* and in *vivo*. | [99] |
| Real-time PCR, Solid-phase ELISA. | 3T3-L1, anti-GLUT4 antibody from Pierce (1:1000). | Treatments without or with the extract. | The crude extract of stevia leaf can enhance *Slc2a4* mRNA and GLUT4 protein levels in 3T3-L1 cells. | [100] |
| GeXP multiplex for mRNA, Western blot. | 3T3-L1, anti-GLUT4 from Millipore (1:20). | Treatments without or with indicated reagents. | Curculigoside and ethyl acetate fractions increase glucose transport activity of 3T3-L1 adipocytes *via* GLUT4 translocation. | [101] |
| Real-time PCR, Western blot. | 3T3-L1, anti-GLUT4 from Cell Signaling Technology. | Treatments without or with luteolin | Luteolin treatment decreases *Slc2a4* mRNA and GLUT4 protein levels in 3T3-L1 cells. | [102] |
| Western blot. | 3T3-L1, anti-GLUT4 from Abcam (ab654-250). | Treatments without or with extract. | Shilianhua extract treatment decreases GLUT4 protein level in 3T3-L1 cells. | [103] |
| Western blot. | 3T3-L1, and male C57BL/6J mice fed a normal-fat or high-fat diet, anti-mouse GLUT4 from AbD SeroTec (1:1000). | Treatments without or with fargesin. | Fargesin treatment increases GLUT4 protein expression in 3T3-L1 cells and adipose tissues of mice. | [104] |
| Western blot. | 3T3-L1, antibody no mentioned. | Treatments without or with phillyrin. | Phillyrin treatment increases the expression levels of GLUT4 protein in 3T3-L1 cells. | [105] |
| Real-time PCR, Western blot. | 3T3-L1, anti-GLUT4 from Santa Cruz Biotechnology. | Treatments without or with 6Hydroxydaidzein. | 6Hydroxydaidzein facilitates GLUT4 protein translocation, but did not affect *Slc2a4* mRNA level in 3T3-L1 cells. | [106] |
| Western blot. | 3T3-L1, and C57BL/6J mice with *SirT1* and *Ampka1* knockdown, anti-GLUT4 from Signalway Antibody. | Treatments without or with indicated reagents. | Seleniumenriched exopolysaccharides produced by Enterobacter cloacae Z0206 increase the expression of GLUT4 protein in mice, but not in 3T3-L1 cells. | [107] |
| Western blot. | 3T3-L1, anti-GLUT4 from Cell Signaling Technology | Treatments without or with extract. | Aspalathin-enriched green rooibos extract increases GLUT4 protein expression in 3T3-L1 cells. | [108] |
| Transient expression of myc-GLUT4-GFP and fluorescence microscopy. | 3T3-L1, fusion protein only. | Treatments without or with indicated reagents. | Gallic acid can increase GLUT4 translocation and glucose uptake in 3T3-L1 cells. | [109] |
| Real-time PCR, Western blot. | 3T3-L1, anti GLUT4 from Santa Cruz Biotechnology (1: 1000). | Treatments without or with Ginsenoside Re. | Ginsenoside Re promotes the translocation of GLUT4 by activating PPAR-γ2. *Slc2a4* mRNA is not affected in 3T3-L1 cells. | [110] |
| Real-time PCR, GLUT4-myc7-GFP from | 3T3-L1 with knockdown of PPARγ, fusion protein. | Cells without or with | Bone morphogenetic proteins 2 and 6 inhibit PPARγ expression, which increases | [111] |

| | | | | |
|---|---|---|---|---|
| retroviral vector, flow cytometry, fluorescence microscopy. | | knockdown. | total GLUT4 levels, but not GLUT4 translocation3T3-L1 cells. | |
| Western blot, real-time PCR. | 3T3-L1, anti-GLUT4 antibody from Santa Cruz Biotechnology (sc-1608). | Treatments without or with pulse or manipulations. | Glucose pulse (25 mM) increases GLUT4 expression. GLUT4 level is partially restored by increasing intracellular NAD/P levels. A liver X receptor element on *Slc2a4* promoter is responsible for glucose-dependent transcription. | [112] |

GLUT4: Glucose transporter 4; AMPK: AMP-activated protein kinase; CEBPA and C/EBP: CCAAT/enhancer-binding protein alpha; ESR1: Estrogen receptor 1.

protein 4C can block GLUT4 translocation. Rac exchange factor 1 activation seems to promote GLUT4 translocation *via* arrangement of actin cytoskeleton. The mechanism of AMPK-mediated GLUT4 translocation in 3T3-L1 adipocytes seems to be distinct from that of insulin-induced one. Future studies are needed to integrate the roles of all these players in the regulation of GLUT4 system in adipocytes.

## GLUT4 IN THE HEART

The heart works constantly to support the blood circulation throughout the lifespan. Cardiomyocytes constantly contract to pump blood, oxygen, metabolic substrates, and hormones to other parts of the body. This requires continuous ATP production for energy supply. The primary fuel for the heart is fatty acids, whereas glucose and lactate contribute to 30% of energy for ATP production[125]. In addition, glucose plays an important role in circumstances like ischemia, increased workload, and pressure overload hypertrophy.

Glucose is transported into cardiac myocytes through GLUTs. GLUT4 represents around 70% of the total glucose transport activities[15]. GLUT4 protein expression can be found as early as 21 days of gestation in rats[126]. The expression level of GLUT4 in the heart may increase or decrease depending on the different models. For example, GLUT4 protein content decreases along with aging in male Fischer rats, but increases 4-5 times in C57 Bl6 mice[127,128]. In basal state, GLUT4 is found mainly in intracellular membrane compartments, and can be stimulated by insulin and ischemia to translocate to the cell membrane[129]. The binding of myocyte enhancer factor-2 (MEF2) and thyroid hormone receptor alpha 1 is needed for transcription of *Slc2a4* gene in cardiac and skeletal muscle in rats[130]. In addition, *Slc2a4* gene expression can also be regulated by other transcription factors. For example, overexpression of peroxisome proliferator-activated receptor gamma coactivator 1 works with MEF2-C to induce *Slc2a4* mRNA expression in L6 muscle cells[131]. Moreover, GLUT4 expression level can be affected by cardiovascular diseases, and myocardial sarcolemma, which reduce the expression and translocation of GLUT4[132]. The development of T1DM decreases GLUT4 expression level and its translocation in the heart of mice[132]. T2DM development also reduces GLUT4 content and translocation due to insulin resistance

**Table 4 Recent studies of mechanisms of glucose transporter 4 expression and translocation in adipocytes**

| Methods | Materials | Comparisons | Observations/conclusions | Ref. |
|---|---|---|---|---|
| Western blot, real-time PCR, Electrophoretic mobility shift assay. | 3T3-L1 pre and differentiated adipocytes. Anti-GLUT4 antibody from Chemicon (1:4,000). | Treatment groups without or with the antagonist. | CB1 receptor antagonist markedly increases *Slc2a4* mRNA and GLUT4 protein levels in 3T3-L1 cells *via* NF-kB and SREBP-1 pathways. | [113] |
| Immunohistochemistry, Western blot, real-time PCR. | Brown adipose tissue of *Arfrp1* flox/flox and *Arfrp1* ad-/- mouse embryos (ED 18.5) and 3T3-L1 cells with knockdown of Arfrp1. Anti-GLUT4 without specifying the vendor (1:1000). | Mice without or with deletion, and 3T3-L1 cells without or with knockdown. | In *Arfrp1* ad-/- adipocytes, GLUT4 protein accumulates on the cell membrane rather than staying intracellularly without any change of *Slc2a4* mRNA. siRNA-mediated knockdown of *Arfrp1* in 3T3-L1 adipocytes has a similar result and increases basal glucose uptake. | [114] |
| Real-time PCR, Western blot. | 3T3-L1 transfected with Mmu-miR-29a/b/c. Anti-GLUT4 from Santa Cruz Biotechnology (SC-7938). | Cells with or without transfection. | Transfection of miR-29 family members inhibits *Slc2a4* mRNA and GLUT4 protein levels in 3T3-L1 cells by inhibiting SPARC expression. | [115] |
| Northern blot, Western blot, Nuclear run-on assay for the rate of GLUT4 gene transcription. | 3T3-L1 pre and differentiated adipocytes. Rabbit polyclonal GLUT4 antibody form Chemicon. | Treatment groups without or with inhibitors. | Inhibitions of proteasome using Lactacystin and MG132 reduce *Slc2a4* mRNA and GLUT4 protein levels in 3T3-L1 cells. | [116] |
| AFFX miRNA expression chips for mRNA, Western blot. | Human Omental adipose tissue, 3T3-L1 pre and differentiated adipocytes with miR-222 silenced by antisense oligonucleotides. Anti-GLUT4 from Abcam. | Groups without or with transfection. | High levels of estrogen reduce the expression and translocation of GLUT4 protein. miR -222 silencing dramatically increases the GLUT4 expression and the insulin-stimulated translocation of GLUT4 in 3T3-L1 adipocytes. | [117] |
| Northern blot for mRNA, Western blot. | 3T3-L1 pre and differentiated adipocytes. Anti-GLUT4 from Chemicon. | Treatment groups without or with oxidative stress. | Oxidative stress mediated by hydrogen peroxide induces expressions of C/EBPα and δ, resulting in altered C/EBP-dimer composition on the GLUT4 promoter, which reduces GLUT4 mRNA and protein levels. | [118] |
| Real-time PCR, Western blot. | Human Subcutaneous pre and differentiated adipocytes from control and obese subjects, 3T3-L1 pre and differentiated adipocytes transfected with miR-155. Anti-GLUT4 from Abcam. | Primary pre and differentiated adipocytes from normal and obese subjects, and cells without or with transfection. | The level of *SLC2A4* is reduced in obese people, and the expression of GLUT4 protein is reduced in 3T3-L1 cells and differentiated human mesenchymal stem cells transfected with miR-155. | [119] |
| HA-GLUT4-GFP from transfected lentiviral plasmid and analyzed by flow cytometry, and fluorescence microscopy. | 3T3-L1 pre and differentiated adipocytes with knockdown of *Dennd4C*. Fusion protein. | Groups without or with knockdown. | Knockdown of *Dennd4C* inhibits GLUT4 translocation, and over- expression of DENND4C slightly stimulates it. DENND4C is found in isolated GLUT4 vesicles. | [120] |
| HA-Glut4-GFP from transfected plasmid, and analyzed by flow cytometry, fluorescence microscopy | 3T3-L1 pre and differentiated adipocytes with AS160 knockdown.Fusion protein. | Groups without or with knockdown. | Akt regulates the rate of vesicle tethering/fusion by regulating the concentration of primed, and fusion-competent GSVs with the plasma membrane, but not changing the intrinsic rate constant for tethering/fusion. | [121] |
| HA-tagged GLUT4 by fluorescence microscopy, Western blots, Immune pulldown. | 3T3-L1 pre and differentiated adipocytes without or with GST-ClipR-59 transfection. Rabbit anti-GLUTlut4 from Millipore; Mouse monoclonal anti-GLUT4 from Cell Signaling Technology. | Pull down antibodies. | By interacting with AS160 and enhancing the association of AS160 with Akt, ClipR-59 promotes phosphorylation of AS160 and GLUT4 membrane translocation. | [122] |
| Transfection of GFP-GLUT4 and indirect immunofluorescence. | 3T3-L1 pre and differentiated adipocytes with siRNA knockdown of P-Rex1. Fusion protein. | Without or with knockdown. | P-Rex1 activates Rac1 in adipocytes, which leads to actin rearrangement, GLUT4 trafficking, increase of glucose uptake. | [123] |
| Transfection of GLUT4-eGFP plasmid and analyzed by fluorescence microscopy. | 3T3-L1 pre and differentiated adipocytes. Fusion protein. | Treatment groups without or with activators. | AMPK-activated GLUT4 translocation in 3T3-L1 adipocytes is mediated through the insulin-signaling pathway distal to the site of activated phosphatidylinositol 3-kinase or through a signaling system distinct from that activated by insulin. | [124] |

GLUT4: Glucose transporter 4; ARFRP1: ADP-ribosylation factor-related protein 1; AMPK: AMP-activated protein kinase; AS160: Akt substrate of 160 kDa; CB1: Cannabinoid receptor 1; CEBPA and C/EBP: CCAAT/enhancer-binding protein alpha; CLIPR-59: Cytoplasmic linker protein R-59; DENND4C: Differentially expressed in normal and neoplastic cells domain-containing protein 4C; GSV: GLUT4 storage vesicle; NF-Kb: Nuclear factor-κB; PREX1: Phosphatidylinositol-3,4,5-trisphosphate dependent Rac exchange factor 1; SPARC: Secreted protein acidic and rich in cysteine; SREBP-1: Sterol regulatory element-binding protein 1.

and impairments of insulin signaling pathway in human cardiomyocytes[133].

To investigate the methods and reagents used for GLUT4 studies in the heart, "GLUT4, heart", and "cardiomyocytes, GLUT4 expression" were used as key words to search PubMed for articles published after 2000. We went through all papers with cardiomyocytes and GLUT4 in titles or short descriptions and selected 9 of them that are mainly focused on GLUT4 expression and translocation in the heart as shown in Table 5[134-142].

Rats are used in all 9 studies. Various methods and reagents are used to analyze *Slc2a4* mRNA and GLUT4 protein levels in the heart and cardiomyocytes. Real-time PCR was used in 4 of them to determine *Slc2a4* mRNA levels. Antibodies and Western blot were used to assess GLUT 4 protein in 8 of them. Immunohistochemistry was used in 1 of them. Two of them used immunofluorescence to track down GLUT4 translocation.

To determine the content of GLUT4 protein in the heart, Western blot and fusion protein immunofluorescence methods were used. As shown in Table 5, these studies do not include overexpressed GLUT4 or cell samples with *Slc2a4* deletion as controls. Several of them did not mention sources of anti GLUT4 antibodies used in Western blots. Some used polyclonal antibodies, which may need a positive control to indicate the correct size and location of GLUT4 protein.

From the papers listed above, GLUT4 expression and translocation in the heart and cardiomyocytes can be affected through activations of ERK and Akt pathways. Proteins like growth hormone, catestatin or pigment epithelium-derived factor can stimulate GLUT4 translocation and glucose uptake. Chemicals like sitagliptin and ethanol can up- and down-regulate *Slc2a4*'s mRNA expression levels, respectively. However, the underlying mechanisms responsible for these regulations of GLUT4 translocation and *Slc2a4* mRNA expression remain to be revealed. In addition, the research results summarized here are from tissue and cells of rats. It will be interesting to see whether same results will be observed when tissues and cells from other animal models are used.

## GLUT4 IN THE BRAIN

The brain is a complex organ in the body and controls a variety of functions from emotions to metabolism. It consists of cerebrum, the brainstem, and the cerebellum[143]. Brain cells utilize glucose constantly to produce energy in normal physiological conditions. The brain can consume about 120 g of glucose per day, which is about 420

**Table 5 Recent studies of glucose transporter 4 expression and translocation in the heart**

| Methods | Materials | Comparisons | Conclusions | Ref. |
|---|---|---|---|---|
| Western blot. | Cytosol and membrane fractions of left ventricular, heart, and blood from male Sprague-Dawley rats. Anti-GLUT4 from Santa Cruz Biotechnology (1:200). | Groups without or with the indicated treatments. Na+/K+-ATPase and β-actin were loading controls of the membrane and cytosol fractions, respectively. Losartan was used as a positive control. | Ginsenoside Rb1 treatment can increase GLUT4 expression *via* inhibition of the TGF-β1/Smad and ERK pathways, and activation of the Akt pathway. | [134] |
| Real-time PCR,Western blot. | Isolated ventricular cells from heart of male adult (aged 6-8 wk) and neonatal (1-3 d old) Wistar rats.Anti GLUT4 from Abcam (unknown dilution). | Groups with or without the ethanol feeding. *Gapdh* and β-actin were included as loading controls for real-time PCR and Western blot, respectively. | Long-term (22 wk) ethanol consumption increases AMPK and MEF2 expressions, and reduces GLUT4 mRNA and protein expression in rat myocardium | [135] |
| Western Blot. | Isolated ventricular cells from heart of adult male Wistar rats. Polyclonal rabbit anti-human GLUT4 from AbD Serotec (4670–1704 1:750) | Groups with or without the indicated treatments. | Heart failure and MI reduce glucose uptake and utilization. GGF2 partially rescues GLUT4 translocation during MI. | [136] |
| Western blot, Immunofluorescence. | Isolated ventricular cells from heart of adult rats. Polyclonal anti-GLUT4 from Thermo Fisher Scientific (1:100). | Treatment groups were compared with that of 100 nM insulin. | Catestatin can induce AKT phosphorylation, stimulate glucose uptake, and increase GLUT4 translocation. | [137] |
| Western blot, Flow cytometric analysis. | Isolated ventricular cells from heart of adult male Wistar rats. Anti-GLUT4 (H-61) from unknow source (1:1000 for Western) and conjugated to Alexa Fluor 488. | Treatment groups with or without AMPK agonists. | AMPK activation does not affect GLUT4 translocation and glucose uptake in isolated cardiomyocytes. | [138] |
| Real-time PCRUsing TaqMan® Gene Expression assays. | Blood, heart, frontal cortex cerebellum from male Wistar rats. | Tissues from control and diabetic rats. | *Slc2a4*'s expression is downregulated in STZ-treated rat's heart, but unaffected in tissue protected by blood-brain barrier like frontal cortex. | [139] |
| Western blot, Immunohistochemistry. | Heart from male Sprague-Dawley rats, anti-GLUT4 from Cell Signaling Technology (2213, 1:1000), anti GLUT4 from Abcam (ab654, 1:200 for ICC/IF) | Treatment groups without or with the indicated treatments. | PEDF can increase glucose uptake and GLUT4 translocation in ischemic myocardium. | [140] |
| Real-time PCR,Western blot. | Heart from male wild type rats and SHRs. Rabbit polyclonal antibody GLUT4 from Millipore | Wild type rats and SHRs without or with the indicated treatments. | Sitagliptin upregulates levels of GLUT4 protein and *Slc2a4* mRNA, and its translocation in cardiac muscles of SHRs. | [141] |
| Real-time PCR,Western blot. | Left ventricles muscle from male Wistar rats. Anti-GLUT4 from Chemicon (1:1000) | Saline as untreated control and reagent treated groups. | Growth hormone stimulates the translocation of GLUT4 to the cell membrane of cardiomyocytes in adult rats. | [142] |

GLUT4: Glucose transporter 4; AMPK: Adenosine monophosphate-activated protein kinase; ERK: Extracellular signal-regulated kinase; GGF2: Glial growth factor 2; GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase; MEF2: Myocyte enhancer factor-2; MI: Myocardial infarction; PEDF: Pigment epithelium-derived factor; SHR: Spontaneously hypertensive rats; STZ: Streptozotocin; TGF: Transforming growth factors.

kcal and accounts for 60% of glucose ingested in a human subject[125]. The glucose influx and metabolism in the brain can be affected by multiple factors such as aging, T2DM and Alzheimer's disease[144]. Reduction of glucose metabolism in the brain can lead to cognitive deficits[145]. Due to the critical relationship between cognitive performance and glucose metabolism, it is important to understand the regulatory mechanism of glucose metabolism in the brain.

Studies have shown that insulin signaling can be impacted in both T2DM and Alzheimer's disease[146,147]. Insulin is a key component for hippocampal memory process, and specifically involved in regulating hippocampal cognitive processes and

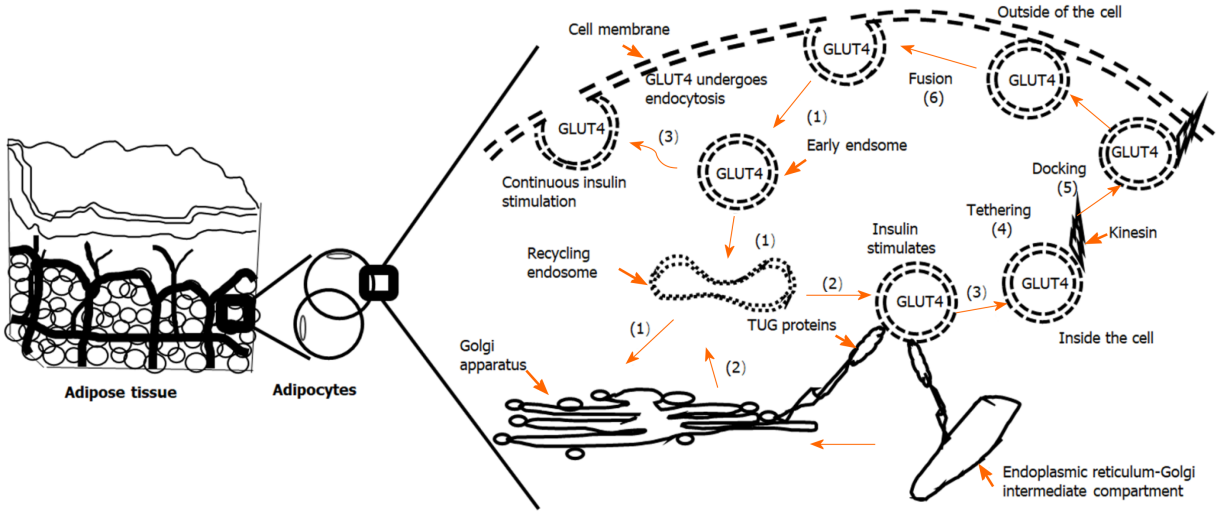


**Figure 2 The movement of glucose transporter 4 in adipocytes.** Adipose tissue is made of adipocytes. In adipocytes, glucose transporter 4 (GLUT4) can be found in the cell membrane and in the cytosol. The translocation of GLUT4 from cytosolic vesicles to the cell membrane leads to elevated glucose uptake, whereas endocytosis brings GLUT4 back to the cytosol. (1): In unstimulated cells, GLUT4 containing membrane portions are internalized in an endocytosis manner to generate vesicles containing GLUT4. GLUT4 vesicles are internalized into early (or sorted) endosomes. They can enter the recovery endoplasmic body, and follow the retrograde pathway to the trans-Golgi network and endoplasmic reticulum-Golgi intermediate compartment or other donor membrane compartments. (2): GLUT4 vesicles derived from the donor membrane structures are secured by tether containing a UBX domain for GLUT4 (TUG) protein. (3): During insulin signal stimulation, GLUT4 vesicles are released and loaded onto the microtubule motor to be transferred to the plasma membrane. The continuous presence of insulin leads to the direct movement of these vesicles to the plasma membrane. (4): GLUT4 vesicles are tethered to motor protein kinesin and other proteins. A stable ternary SNARE complex forms when this occurs. (5): The stable ternary SNARE complex is docked on the target membrane. (6): The docked vesicles rely on SNARE to move to and fuse with the target membrane[60,90,94]. GLUT4: Glucose transporter 4.

metabolism[148]. Insulin-modulated glucose metabolism depends on regions in the brain. The cortex and hippocampus are the most sensitive areas in the brain[149]. Hippocampus located deeply in temporal lobe plays an important role in learning and memory, and relates to diseases like Alzheimer's disease, short term memory loss and disorientation[150]. Hippocampal cognitive and metabolic impairments are relatively common in T2DM, which may be caused by diet-induced obesity and systemic insulin resistance[151]. On the other hand, insulin stimulation can enhance memory and cognitive function[152]. This enhancement may require the brain GLUT4 translocation as shown in rats[153]. It is very important to determine the expression profile of GLUT4 in the brain, and factors that impact GLUT4 expression and translocation.

To summarize methods and reagents used in brain GLUT4 studies, "brain, GLUT4 expression" were used as key words to search PubMed for articles that have brain and GLUT4 in their titles or short descriptions. Ten research articles published after 2000 were identified as representative ones, which are focused on GLUT4 translocations and content in the brain of rats and mice. We summarized the methods and reagents for GLUT4 analysis, and conclusions as shown in Table 6[154-163].

In these 10 papers, two of them used real-time PCR to determine the *Slc2a4* mRNA in the brain. Eight of them used anti-GLUT4 antibodies and Western blot to detect GLUT 4 protein. Five papers included β-actin as loading control in Western blot. Four used immunohistochemistry. One paper used electrophysiological technique, and one paper used fluorescent microscopy to identify GLUT 4 in neurons. One study used brain specific *Slc2a4* knockout and wild type mice to study the functions of GLUT4 in the brain.

In conclusion, results of Western blot and real-time PCR demonstrate that GLUT4 protein and *Slc2a4* mRNA can be detected in rat's brain and central nervous system. Deletion of *Slc2a4* in the brain causes insulin resistance, glucose intolerance, and impaired glucose sensing in the ventromedial hypothalamus. GLUT4 mediates the effects of insulin, or insulin-like growth factor on regulations of cognition, memory, behavior, motor activity and seizures. GLUT4 positive neurons are responsible for glucose sensing. Physical activity improves GLUT4 translocation in neurons, a process that needs Rab10 phosphorylation. Interestingly, 27-OH cholesterol treatment seems to decrease GLUT4 expression in the brain. Studies of *Slc2a4* mRNA and GLUT4 protein in the brain and central nervous system have begun to demonstrate the potential roles of GLUT4 expression and its translocation in the regulation of glucose metabolism in

**Table 6 Recent studies of glucose transporter 4 expression and translocation in the brain**

| Methods | Materials | Comparisons | Conclusions | Ref. |
|---|---|---|---|---|
| Western blot. | Brain, skeletal muscle, heart, and whiteadipose tissue from mice. Anti-GLUT4 from Chemicon (1:1000). | Samples from wild type and knockout mice. | Deletion of *Slc2a4* in the brain leads to insulin resistance, glucose intolerance, and impaired glucose sensing in the VMH. | [154] |
| Western blot,Real time-PCR,Immunofluorescence. | Cortex, hypothalamus, cerebella samples from CD-1 mice. Monoclonal anti Glut4 (1F8) from Dr. Paul Pilch, Polyclonal anti Glut4 MC2A from Dr. Giulia Baldini, Polyclonal anti Glut4 αG4 from Dr. Samuel Cushman, Polyclonal anti Glut4 (C-20) from Santa Cruz Biotechnology. | Expression profile in the mouse and rat brain samples. | *Slc2a4* mRNA is expressed in cultured neurons. GLUT4 protein is highly expressed in the granular layer of the mouse cerebellum. GLUT4 translocation to the plasma membrane can be stimulated by physical activity. | [155] |
| Western blot. | Brian tissue from STZ-induced diabetic male Sprague-Dawley rats.Anti-GLUT4 from Millipore (1:1000). | Comparing treatment samples using β-actin and NA/K ATPase as loading controls in Western blot. | Chronic infusion of insulin into the VMH in poorly controlled diabetes is sufficient to normalize the sympathoadrenal response to hypoglycemia *via* restoration of GLUT4 expression. | [156] |
| Immunocytochemistry. | Cerebellum and hippocampus from male Sprague-Dawley rats.Rabbit anti-GLUT4 antibody from Alomone Labs (AGT-024, RRID: AB_2631197). | Identifying expression profile and translocation. | GLUT4 is expressed in neurons including nerve terminals.Exercising axons rely on translocation of GLUT4 to the cell membrane for metabolic homeostasis. | [157] |
| Real-time PCR, Immunocytochemistry. | Cerebral cortex, hippocampus, thalamus, cerebellum, medulla oblongata, cervical spinal cord, biceps muscles from male Wistar rats. Unknown source of antibody. | Identifying expression profile using β -actin as loading control in real-time PCR. | *Slc2a4* mRNA is detected in many neurons located in brain and spinal cord. GLUT4 protein is detected in different regions of the CNS including certain allocortical regions, temporal lobe, hippocampus, and substantia nigra. | [158] |
| Immunocytochemistry, Western blot. | Brain, spleen, kidney from *Lrrk2* knockout mice.Anti GLUT4 Avivasysbio (ARP43785_P050, 1:100), andanti GLUT4 from R&D Systems (MAB1262, 1:1000). | Samples from wild type and knockout mice, andanti-β-Tubulin as loading control. | Phosphorylation of Rab10 by LRRK2 is necessary for GLUT4 translocation.*Lrrk2* deficiency increases GLUT4 expression on the cell surface in "aged" cells. | [159] |
| Western blot, Immunofluorescence, real-time PCR. | Brain from Cyp27Tg mice. Anti GLUT4 from Cell Signaling Technology (#2213,1:1000 dilution). | Mice with different treatments. | A reduction of GLUT4 protein expression in brain occurs after 27-OH cholesterol treatment. | [160] |
| Immunohistochemistry. | Brain, hypothalamus, and other tissues from Sprague–Dawley rats.Anti-GLUT4 antibody from Santa Cruz Biochemicals (1:200), Anti GLUT4 from S. Cushman (1:1000). | Identifying the expression profiles. Soleus muscle as GLUT4 positive control. Antibodies after pre-absorption with the corresponding synthetic peptide were used as negative control. for GLUT4 antibody. | GLUT4 is localized to the micro vessels comprising the blood brain barrier of the rat VMH.GLUT4 is co-expressed with both GLUT1 and zonula occludens-1 on the endothelial cells of these capillaries. | [161] |
| Electrophysiological analyses, fluorescent microscope. | Brain from GLUT4-EYFP transgenic mice. Fusion protein. | Comparing samples from treatments. A scrambled RNA expressed by AAV acted as a negative control. | GLUT4 neurons are responsible for glucose sensing. | [162] |
| Western blot,Immunohistochemistry. | Brain samples from 7, 11, 15, 21 and 60 d old Balb/c mice. Rabbit anti-rat GLUT4 from an unknow source (1:2500 dilution for Western and 1:2000 for immunohistochemistry). | Determine the expression profiles. Vinculin is used as the loading control in Western blot. | GLUT4 is expressed in neurons of the postnatal mouse brain. GLUT4 and GLUT8 may mediate the effects of insulin, or insulin-like growth factor on regulations of cognition, memory, behavior, motor activity and seizures. | [163] |

AAV: Adeno-associated virus; CNS: Central nervous system; CRR: Counterregulatory response; EYFP: Enhanced Yellow Fluorescent Protein; LRRK2: Leucine-rich repeat kinase 2; STZ: Streptozotocin; VMH: Ventromedial hypothalamus.

the brain and central nervous system. More studies of GLUT4 expression and translocation in the control of functions and metabolism in various region in the brain

and central nervous system are expected in the future.

## CONCLUSION

GLUT4 is generally thought to contribute to insulin-stimulated glucose uptake in adipocytes and skeletal muscle. Studies summarized here seem to show that GLUT4 is also expressed in the brain, neurons, and heart. GLUT4 is expressed concurrently with other GLUTs in multiple tissues in a temporal and spatial specific manner such as during brain development[163]. Hormones and cytokines other than insulin can also regulate the expression levels and translocation of GLUT4 in different tissues[141,142,163]. In adipocytes alone, many bioactive compounds or chemical reagents have shown to affect GLUT4 pathways as shown in Table 3. All these seem to indicate that the regulatory mechanism of the GLUT4 pathway is complicated than we originally proposed.

So far, various methods from gene knockout to immunohistochemistry have been used to study the mechanisms of *Slc2a4* mRNA and GLUT4 expressions, and its translocation in different cells. Every technique has its pros and cons. Based on the studies summarized here, anti-GLUT4 antibodies from a variety of sources have been used to study GLUT4 expression and translocation. The conclusions of these studies are based on the experimental results derived from the use of those antibodies. A positive control derived from a cell or tissue with unique overexpression or silencing of GLUT4 is critical to confirm the antibody's specificity to pick up a right signaling in the study system. This is especially true for Western blot. It appears that some of the studies did not include control groups like this. Another challenge facing biochemical study of GLUT4 translocation in the muscle may be the sample processing. This probably explains why fusion proteins and stable cell lines are developed to enhance signals and specificities for detection. Confirmation of the antibody specificity in a particular system probably should be done first.

As glucose homeostasis is a complicate process involved in many players. It is anticipated to see that many proteins seem to play a role in the regulation of GLUT4 system. It will be interesting to see how GLUT4 in different regions of the brain contributes to the regulation of glucose metabolism, and what the roles of insulin-induced GLUT4 translocation in those areas are. In addition, other GLUTs are also expressed in the same cells that GLUT4 are expressed. How GLUT4 works with other GLUTs to regulate metabolism also deserves to be investigated. Last, as glucose usage in the skeletal muscle is altered in insulin resistance and T2DM, how GLUT4 system contributes to progressions and interventions of these diseases still remains to be the focus in the future. Nevertheless, further understanding GLUT4 system will be very helpful for us to combat the development of T2DM.

## ACKNOWLEDGEMENTS

We would like to thank the Department of Nutrition at the University of Tennessee at Knoxville for financial support to T.W. G.C. would like to thank Yantai Zestern Biotechnique Co. LTD for the research funding support.

## REFERENCES

1 **Al-Lawati JA**. Diabetes Mellitus: A Local and Global Public Health Emergency! *Oman Med J* 2017; **32**: 177-179 [PMID: 28584596 DOI: 10.5001/omj.2017.34]

2 **La Flamme KE**, Mor G, Gong D, La Tempa T, Fusaro VA, Grimes CA, Desai TA. Nanoporous alumina capsules for cellular macroencapsulation: transport and biocompatibility. *Diabetes Technol Ther* 2005; **7**: 684-694 [PMID: 16241869 DOI: 10.1089/dia.2005.7.684]

3 **Salt IP**, Johnson G, Ashcroft SJ, Hardie DG. AMP-activated protein kinase is activated by low glucose in cell lines derived from pancreatic beta cells, and may regulate insulin release. *Biochem J* 1998; **335** (Pt 3): 533-539 [PMID: 9794792 DOI: 10.1042/bj3350533]

4 **Stipanuk MH**, Caudill MA. Biochemical, Physiological, and Molecular Aspects of Human Nutrition. 3rd ed. St. Louis, MO: Elsevier, 2012: 968

5 **Quistgaard EM**, Löw C, Guettou F, Nordlund P. Understanding transport by the major facilitator superfamily (MFS): structures pave the way. *Nat Rev Mol Cell Biol* 2016; **17**: 123-132 [PMID: 26758938 DOI: 10.1038/nrm.2015.25]

6 **Klip A**, McGraw TE, James DE. Thirty sweet years of GLUT4. *J Biol Chem* 2019; **294**: 11369-11381 [PMID: 31175156 DOI: 10.1074/jbc.REV119.008351]

7 **Richter EA**, Derave W, Wojtaszewski JF. Glucose, exercise and insulin: emerging concepts. *J Physiol* 2001; **535**: 313-322 [PMID: 11533125 DOI: 10.1111/j.1469-7793.2001.t01-2-00313.x]

8 **Cushman SW**, Wardzala LJ. Potential mechanism of insulin action on glucose transport in the isolated rat adipose cell. Apparent translocation of intracellular transport systems to the plasma membrane. *J Biol Chem* 1980; **255**: 4758-4762 [PMID: 6989818 DOI: 10.1093/aje/kwm394]

9 **Suzuki K**, Kono T. Evidence that insulin causes translocation of glucose transport activity to the plasma membrane from an intracellular storage site. *Proc Natl Acad Sci* **77**: 2542-2545 [PMID: 6771756 DOI: 10.1073/pnas.77.5.2542]

10 **Wardzala LJ**, Cushman SW, Salans LB. Mechanism of insulin action on glucose transport in the isolated rat adipose cell. Enhancement of the number of functional transport systems. *J Biol Chem* 1978; **253**: 8002-8005 [PMID: 711732]

11 **Klip A**, Ramlal T, Young DA, Holloszy JO. Insulin-induced translocation of glucose transporters in rat hindlimb muscles. *FEBS Lett* 1987; **224**: 224-230 [PMID: 2960560 DOI: 10.1016/0014-5793(87)80452-0]

12 **Wardzala LJ**, Jeanrenaud B. Potential mechanism of insulin action on glucose transport in the isolated rat diaphragm. Apparent translocation of intracellular transport units to the plasma membrane. *J Biol Chem* 1981; **256**: 7090-7093 [PMID: 6265437]

13 **James DE**, Brown R, Navarro J, Pilch PF. Insulin-regulatable tissues express a unique insulin-sensitive glucose transport protein. *Nature* 1988; **333**: 183-185 [PMID: 3285221 DOI: 10.1038/333183a0]

14 **Olson AL**. Regulation of GLUT4 and Insulin-Dependent Glucose Flux. *ISRN Mol Biol* 2012; **2012**: 856987 [PMID: 27335671 DOI: 10.5402/2012/856987]

15 **Mueckler M**, Thorens B. The SLC2 (GLUT) family of membrane transporters. *Mol Aspects Med* 2013; **34**: 121-138 [PMID: 23506862 DOI: 10.1016/j.mam.2012.07.001]

16 **Chen L**, Tuo B, Dong H. Regulation of Intestinal Glucose Absorption by Ion Channels and Transporters. *Nutrients* 2016; **8** [PMID: 26784222 DOI: 10.3390/nu8010043]

17 **Navale AM**, Paranjape AN. Glucose transporters: physiological and pathological roles. *Biophys Rev* 2016; **8**: 5-9 [PMID: 28510148 DOI: 10.1007/s12551-015-0186-2]

18 **Grefner NM**, Gromova LV, Gruzdkov AA, Komissarchik YY. Interaction of glucose transporters SGLT1 and GLUT2 with cytoskeleton in enterocytes and Caco2 cells during hexose absorption. *Cell and Tissue Biol* 2015; **9**: 45-52 [DOI: 10.1134/S1990519X15010034]

19 **Gropper S**, Smith J, Carr T. Advanced Nutrition and Human Metabolism. 7th ed. Publisher: Wadsworth Publishing, 2016: 640

20 **Bell GI**, Kayano T, Buse JB, Burant CF, Takeda J, Lin D, Fukumoto H, Seino S. Molecular biology of mammalian glucose transporters. *Diabetes Care* 1990; **13**: 198-208 [PMID: 2407475 DOI: 10.2337/diacare.13.3.198]

21 **Haber RS**, Weinstein SP, O'Boyle E, Morgello S. Tissue distribution of the human GLUT3 glucose transporter. *Endocrinology* 1993; **132**: 2538-2543 [PMID: 8504756 DOI: 10.1210/endo.132.6.8504756]

22 **Stuart CA**, Wen G, Gustafson WC, Thompson EA. Comparison of GLUT1, GLUT3, and GLUT4 mRNA and the subcellular distribution of their proteins in normal human muscle. *Metabolism* 2000; **49**: 1604-1609 [PMID: 11145124 DOI: 10.1053/meta.2000.18559]

23 **Lisinski I**, Schürmann A, Joost HG, Cushman SW, Al-Hasani H. Targeting of GLUT6 (formerly GLUT9) and GLUT8 in rat adipose cells. *Biochem J* 2001; **358**: 517-522 [PMID: 11513753 DOI: 10.1042/0264-6021:3580517]

24 **Takata K**, Kasahara T, Kasahara M, Ezaki O, Hirano H. Erythrocyte/HepG2-type glucose transporter is concentrated in cells of blood-tissue barriers. *Biochem Biophys Res Commun* 1990; **173**: 67-73 [PMID: 2256938 DOI: 10.1016/S0006-291X(05)81022-8]

25 **Joost HG**, Thorens B. The extended GLUT-family of sugar/polyol transport facilitators: nomenclature, sequence characteristics, and potential function of its novel members (review). *Mol Membr Biol* 2001; **18**: 247-256 [PMID: 11780753 DOI: 10.1080/09687680110090456]

26 **Zhao FQ**, Keating AF. Functional properties and genomics of glucose transporters. *Curr Genomics* 2007; **8**: 113-128 [PMID: 18660845 DOI: 10.2174/138920207780368187]

27 27 Szablewski L. Glucose Homeostasis and Insulin Resistance. Bentham Science Publishers, 2011: 211

28 **Hruz PW**, Mueckler MM. Structural analysis of the GLUT1 facilitative glucose transporter (review). *Mol Membr Biol* 2001; **18**: 183-193 [PMID: 11681785 DOI: 10.1080/09687680110072140]

29 **Cheeseman C**, Long WL. Structure of, and functional insight into the GLUT family of membrane transporters. *Cell Health Cytoskeleton* 2015; **7**: 167 [DOI: 10.2147/chc.s60484]

30 **Olson AL**, Pessin JE. Structure, function, and regulation of the mammalian facilitative glucose transporter gene family. *Annu Rev Nutr* 1996; **16**: 235-256 [PMID: 8839927 DOI: 10.1146/annurev.nu.16.070196.001315]

31 **Uldry M**, Ibberson M, Hosokawa M, Thorens B. GLUT2 is a high affinity glucosamine transporter. *FEBS Lett* 2002; **524**: 199-203 [PMID: 12135767 DOI: 10.1016/s0014-5793(02)03058-2]

32 **Johnson JH**, Newgard CB, Milburn JL, Lodish HF, Thorens B. The high Km glucose transporter of islets of Langerhans is functionally similar to the low affinity transporter of liver and has an identical primary sequence. *J Biol Chem* 1990; **265**: 6548-6551 [PMID: 2182619 DOI: 10.1016/0008-6215(90)84177-V]

33 **Colville CA**, Seatter MJ, Jess TJ, Gould GW, Thomas HM. Kinetic analysis of the liver-type (GLUT2) and brain-type (GLUT3) glucose transporters in Xenopus oocytes: substrate specificities and effects of transport inhibitors. *Biochem J* 1993; **290** (Pt 3): 701-706 [PMID: 8457197 DOI: 10.1042/bj2900701]

34 **Longo N**, Elsas LJ. Human glucose transporters. *Adv Pediatr* 1998; **45**: 293-313 [PMID: 9742306]

35 **Kayano T**, Fukumoto H, Eddy RL, Fan YS, Byers MG, Shows TB, Bell GI. Evidence for a family of human glucose transporter-like proteins. Sequence and gene localization of a protein expressed in fetal skeletal muscle and other tissues. *J Biol Chem* 1988; **263**: 15245-15248 [PMID: 3170580]

36 **Kasahara T**, Kasahara M. Characterization of rat Glut4 glucose transporter expressed in the yeast Saccharomyces cerevisiae: comparison with Glut1 glucose transporter. *Biochim Biophys Acta* 1997; **1324**: 111-119 [PMID: 9059504 DOI: 10.1016/S0005-2736(96)00217-9]

37 **Fukumoto H**, Kayano T, Buse JB, Edwards Y, Pilch PF, Bell GI, Seino S. Cloning and characterization of the major insulin-responsive glucose transporter expressed in human skeletal muscle and other insulin-responsive tissues. *J Biol Chem* 1989; **264**: 7776-7779 [PMID: 2656669 DOI: 10.1111/j.1432-1033.1989.tb14792.x]

38 **Li Q**, Manolescu A, Ritzel M, Yao S, Slugoski M, Young JD, Chen XZ, Cheeseman CI. Cloning and functional characterization of the human GLUT7 isoform SLC2A7 from the small intestine. *Am J Physiol Gastrointest Liver Physiol* 2004; **287**: G236-G242 [PMID: 15033637 DOI: 10.1152/ajpgi.00396.2003]

39 **Burant CF**, Takeda J, Brot-Laroche E, Bell GI, Davidson NO. Fructose transporter in human spermatozoa and small intestine is GLUT5. *J Biol Chem* 1992; **267**: 14523-14526 [PMID: 1634504]

40 **Drozdowski LA**, Thomson AB. Intestinal sugar transport. *World J Gastroenterol* 2006; **12**: 1657-1670 [PMID: 16586532 DOI: 10.3748/wjg.v12.i11.1657]

41 **Douard V**, Ferraris RP. Regulation of the fructose transporter GLUT5 in health and disease. *Am J Physiol Endocrinol Metab* 2008; **295**: E227-E237 [PMID: 18398011 DOI: 10.1152/ajpendo.90245.2008]

42 **Rand EB**, Depaoli AM, Davidson NO, Bell GI, Burant CF. Sequence, tissue distribution, and functional characterization of the rat fructose transporter GLUT5. *Am J Physiol* 1993; **264**: G1169-G1176 [PMID: 8333543 DOI: 10.1152/ajpgi.1993.264.6.G1169]

43 **Doege H**, Bocianski A, Joost HG, Schürmann A. Activity and genomic organization of human glucose transporter 9 (GLUT9), a novel member of the family of sugar-transport facilitators predominantly expressed in brain and leucocytes. *Biochem J* 2000; **350** Pt 3: 771-776 [PMID: 10970791 DOI: 10.1042/0264-6021:3500771]

44 **Schmidt S**, Joost HG, Schürmann A. GLUT8, the enigmatic intracellular hexose transporter. *Am J Physiol Endocrinol Metab* 2009; **296**: E614-E618 [PMID: 19176349 DOI: 10.1152/ajpendo.91019.2008]

45 **Ibberson M**, Uldry M, Thorens B. GLUTX1, a novel mammalian glucose transporter expressed in the central nervous system and insulin-sensitive tissues. *J Biol Chem* 2000; **275**: 4607-4612 [PMID: 10671487 DOI: 10.1074/jbc.275.7.4607]

46 **Phay JE**, Hussain HB, Moley JF. Cloning and expression analysis of a novel member of the facilitative glucose transporter family, SLC2A9 (GLUT9). *Genomics* 2000; **66**: 217-220 [PMID: 10860667 DOI: 10.1006/geno.2000.6195]

47 **Augustin R**, Carayannopoulos MO, Dowd LO, Phay JE, Moley JF, Moley KH. Identification and characterization of human glucose transporter-like protein-9 (GLUT9): alternative splicing alters trafficking. *J Biol Chem* 2004; **279**: 16229-16236 [PMID: 14739288 DOI: 10.1074/jbc.M312226200]

48 **Dawson PA**, Mychaleckyj JC, Fossey SC, Mihic SJ, Craddock AL, Bowden DW. Sequence and functional analysis of GLUT10: a glucose transporter in the Type 2 diabetes-linked region of chromosome 20q12-13.1. *Mol Genet Metab* 2001; **74**: 186-199 [PMID: 11592815 DOI: 10.1006/mgme.2001.3212]

49 **McVie-Wylie AJ**, Lamson DR, Chen YT. Molecular cloning of a novel member of the GLUT family of transporters, SLC2a10 (GLUT10), localized on chromosome 20q13.1: a candidate gene for NIDDM susceptibility. *Genomics* 2001; **72**: 113-117 [PMID: 11247674 DOI: 10.1006/geno.2000.6457]

50 **Doege H**, Bocianski A, Scheepers A, Axer H, Eckel J, Joost HG, Schürmann A. Characterization of human glucose transporter (GLUT) 11 (encoded by SLC2A11), a novel sugar-transport facilitator specifically expressed in heart and skeletal muscle. *Biochem J* 2001; **359**: 443-449 [PMID: 11583593 DOI: 10.1042/0264-6021:3590443]

51 **Scheepers A**, Schmidt S, Manolescu A, Cheeseman CI, Bell A, Zahn C, Joost HG, Schürmann A. Characterization of the human SLC2A11 (GLUT11) gene: alternative promoter usage, function, expression, and subcellular distribution of three isoforms, and lack of mouse orthologue. *Mol Membr Biol* 2005; **22**: 339-351 [PMID: 16154905 DOI: 10.1080/09687860500166143]

52 **Wu X**, Li W, Sharma V, Godzik A, Freeze HH. Cloning and characterization of glucose transporter 11, a novel sugar transporter that is alternatively spliced in various tissues. *Mol Genet Metab* 2002; **76**: 37-45 [PMID: 12175779 DOI: 10.1016/s1096-7192(02)00018-5]

53 **Manolescu AR**, Witkowska K, Kinnaird A, Cessford T, Cheeseman C. Facilitated hexose transporters: new perspectives on form and function. *Physiology (Bethesda)* 2007; **22**: 234-240 [PMID: 17699876 DOI: 10.1152/physiol.00011.2007]

54 **Sasaki T**, Minoshima S, Shiohama A, Shintani A, Shimizu A, Asakawa S, Kawasaki K, Shimizu N. Molecular cloning of a member of the facilitative glucose transporter gene family GLUT11 (SLC2A11) and identification of transcription variants. *Biochem Biophys Res Commun* 2001; **289**: 1218-1224 [PMID: 11741323 DOI: 10.1006/bbrc.2001.6101]

55 **Rogers S**, Macheda ML, Docherty SE, Carty MD, Henderson MA, Soeller WC, Gibbs EM, James DE, Best JD. Identification of a novel glucose transporter-like protein-GLUT-12. *Am J Physiol Endocrinol Metab* 2002; **282**: E733-E738 [PMID: 11832379 DOI: 10.1152/ajpendo.2002.282.3.E733]

56 **Uldry M**, Ibberson M, Horisberger JD, Chatton JY, Riederer BM, Thorens B. Identification of a mammalian H(+)-myo-inositol symporter expressed predominantly in the brain. *EMBO J* 2001; **20**: 4467-4477 [PMID: 11500374 DOI: 10.1093/emboj/20.16.4467]

57 **Wu X**, Freeze HH. GLUT14, a duplicon of GLUT3, is specifically expressed in testis as alternative splice forms. *Genomics* 2002; **80**: 553-557 [PMID: 12504846 DOI: 10.1006/geno.2002.7010]

58 **Vargas E**, Podder V, Carrillo Sepulveda MA. Physiology, Glucose Transporter Type 4 (GLUT4) 2020 [PMID: 30726007]

59 **Grunberger G**, ZickY. Insulin Signaling: From Cultured Cells to Animal Models. Taylor & Francis, 2003: 448

60 **Bryant NJ**, Govers R, James DE. Regulated transport of the glucose transporter GLUT4. *Nat Rev Mol Cell Biol* 2002; **3**: 267-277 [PMID: 11994746 DOI: 10.1038/NRM782]

61 **Watson RT**, Pessin JE. GLUT4 translocation: the last 200 nanometers. *Cell Signal* 2007; **19**: 2209-2217 [PMID: 17629673 DOI: 10.1016/j.cellsig.2007.06.003]

62 **Stöckli J**, Fazakerley DJ, James DE. GLUT4 exocytosis. *J Cell Sci* 2011; **124**: 4147-4159 [PMID: 22247191 DOI: 10.1242/jcs.097063]

63 **Blot V**, McGraw TE. GLUT4 is internalized by a cholesterol-dependent nystatin-sensitive mechanism inhibited by insulin. *EMBO J* 2006; **25**: 5648-5658 [PMID: 17139247 DOI: 10.1038/sj.emboj.7601462]

64 **Govers R**, Coster AC, James DE. Insulin increases cell surface GLUT4 levels by dose dependently discharging GLUT4 into a cell surface recycling pathway. *Mol Cell Biol* 2004; **24**: 6456-6466 [PMID: 15226445 DOI: 10.1128/MCB.24.14.6456-6466.2004]

65 **Alberts B**, Johnson A, Lewis J, Raff M, Roberts K, Walter P. Molecular Biology of the Cell. 4th ed. New York: Garland Science, 2002: 45-47

66 **Sinacore DR**, Gulve EA. The role of skeletal muscle in glucose transport, glucose homeostasis, and insulin resistance: implications for physical therapy. *Phys Ther* 1993; **73**: 878-891 [PMID: 8248296 DOI: 10.1093/ptj/73.12.878]

67 **Kim KM**, Jang HC, Lim S. Differences among skeletal muscle mass indices derived from height-, weight-, and body mass index-adjusted models in assessing sarcopenia. *Korean J Intern Med* 2016; **31**: 643-650 [PMID: 27334763 DOI: 10.3904/kjim.2016.015]

68 **Daugaard JR**, Richter EA. Relationship between muscle fibre composition, glucose transporter protein 4 and exercise training: possible consequences in non-insulin-dependent diabetes mellitus. *Acta Physiol Scand* 2001; **171**: 267-276 [PMID: 11412139 DOI: 10.1046/j.1365-201x.2001.00829.x]

69 **Brook MS**, Wilkinson DJ, Phillips BE, Perez-Schindler J, Philp A, Smith K, Atherton PJ. Skeletal muscle homeostasis and plasticity in youth and ageing: impact of nutrition and exercise. *Acta Physiol (Oxf)* 2016; **216**: 15-41 [PMID: 26010896 DOI: 10.1111/apha.12532]

70 **Nedachi T**, Kanzaki M. Regulation of glucose transporters by insulin and extracellular glucose in C2C12 myotubes. *Am J Physiol Endocrinol Metab* 2006; **291**: E817-E828 [PMID: 16735448 DOI: 10.1152/ajpendo.00194.2006]

71 **Stanford KI**, Goodyear LJ. Exercise and type 2 diabetes: molecular mechanisms regulating glucose uptake in skeletal muscle. *Adv Physiol Educ* 2014; **38**: 308-314 [PMID: 25434013 DOI: 10.1152/advan.00080.2014]

72 **Nishiumi S**, Ashida H. Rapid preparation of a plasma membrane fraction from adipocytes and muscle cells: application to detection of translocated glucose transporter 4 on the plasma membrane. *Biosci Biotechnol Biochem* 2007; **71**: 2343-2346 [PMID: 17827673 DOI: 10.1271/bbb.70342]

73 **Yamamoto N**, Yamashita Y, Yoshioka Y, Nishiumi S, Ashida H. Rapid Preparation of a Plasma Membrane Fraction: Western Blot Detection of Translocated Glucose Transporter 4 from Plasma Membrane of Muscle and Adipose Cells and Tissues. *Curr Protoc Protein Sci* 2016; **85**: 29.18.1-29.18. 12 [PMID: 27479506 DOI: 10.1002/cpps.13]

74 **Snook LA**, Nelson EM, Dyck DJ, Wright DC, Holloway GP. Glucose-dependent insulinotropic polypeptide directly induces glucose transport in rat skeletal muscle. *Am J Physiol Regul Integr Comp Physiol* 2015; **309**: R295-R303 [PMID: 26041107 DOI: 10.1152/ajpregu.00003.2015]

75 **Takahashi Y**, Kushiro M, Shinohara K, Ide T. Dietary conjugated linoleic acid reduces body fat mass and affects gene expression of proteins regulating energy metabolism in mice. *Comp Biochem Physiol B Biochem Mol Biol* 2002; **133**: 395-404 [PMID: 12431407 DOI: 10.1016/s1096-4959(02)00164-1]

76 **Thomas-Delloye V**, Marmonier F, Duchamp C, Pichon-Georges B, Lachuer J, Barré H, Crouzoulon G. Biochemical and functional evidences for a GLUT-4 homologous protein in avian skeletal muscle. *Am J Physiol* 1999; **277**: R1733-R1740 [PMID: 10600921 DOI: 10.1152/ajpregu.1999.277.6.R1733]

77 **Etgen GJ Jr**, Zavadoski WJ, Holman GD, Gibbs EM. Insulin-sensitive regulation of glucose transport and GLUT4 translocation in skeletal muscle of GLUT1 transgenic mice. *Biochem J* 1999; **337** ( Pt 1): 51-57 [PMID: 9854024 DOI: 10.1042/0264-6021:3370051]

78 **Ploug T**, van Deurs B, Ai H, Cushman SW, Ralston E. Analysis of GLUT4 distribution in whole skeletal muscle fibers: identification of distinct storage compartments that are recruited by insulin and muscle contractions. *J Cell Biol* 1998; **142**: 1429-1446 [PMID: 9744875 DOI: 10.1083/jcb.142.6.1429]

79 **Kristiansen S**, Nielsen JN, Bourgoin S, Klip A, Franco M, Richter EA. GLUT-4 translocation in skeletal muscle studied with a cell-free assay: involvement of phospholipase D. *Am J Physiol Endocrinol Metab* 2001; **281**: E608-E618 [PMID: 11500317 DOI: 10.1152/ajpendo.2001.281.3.E608]

80 **Kawanaka K**, Tabata I, Katsuta S, Higuchi M. Changes in insulin-stimulated glucose transport and GLUT-4 protein in rat skeletal muscle after training. *J Appl Physiol (1985)* 1997; **83**: 2043-2047 [PMID: 9390979 DOI: 10.1152/jappl.1997.83.6.2043]

81 **Rudich A**, Konrad D, Török D, Ben-Romano R, Huang C, Niu W, Garg RR, Wijesekara N, Germinario RJ, Bilan PJ, Klip A. Indinavir uncovers different contributions of GLUT4 and GLUT1 towards glucose uptake in muscle and fat cells and tissues. *Diabetologia* 2003; **46**: 649-658 [PMID: 12712244 DOI: 10.1007/s00125-003-1080-1]

82 **Wozniak SE**, Gee LL, Wachtel MS. Adipose tissue: the new endocrine organ? *Xiaohua Jibing Yu Kexue* 2009; **54**: 1847-56 [DOI: 10.1007/s10620-008-0585-3]

83 **Kershaw EE**, Flier JS. Adipose tissue as an endocrine organ. *J Clin Endocrinol Metab* 2004; **89**: 2548-2556 [PMID: 15181022 DOI: 10.1210/jc.2004-0395]

84 **Li XW**, Huang M, Lo K, Chen WL, He YY, Xu Y, Zheng H, Hu H, Wang J. Anti-Diabetic Effect of a Shihunine-Rich Extract of *Dendrobium loddigesii* on 3T3-L1 Cells and db/db Mice by Up-Regulating AMPK-GLUT4-PPARα. *Molecules* 2019; **24** [PMID: 31340585 DOI: 10.3390/molecules24142673]

85 **Lopez JA**, Burchfield JG, Blair DH, Mele K, Ng Y, Vallotton P, James DE, Hughes WE. Identification of a distal GLUT4 trafficking event controlled by actin polymerization. *Mol Biol Cell* 2009; **20**: 3918-3929 [PMID: 19605560 DOI: 10.1091/mbc.e09-03-0187]

86 **Nishida M**, Funahashi T, Shimomura I. Pathophysiological significance of adiponectin. *Med Mol Morphol* 2007; **40**: 55-67 [PMID: 17572841 DOI: 10.1007/s00795-007-0366-7]

87 **Pond CM**, Mattacks CA. The anatomy of adipose tissue in captive Macaca monkeys and its implications for human biology. *Folia Primatol (Basel)* 1987; **48**: 164-185 [PMID: 2965088 DOI: 10.1159/000156293]

88 **Giordano A**, Smorlesi A, Frontini A, Barbatelli G, Cinti S. White, brown and pink adipocytes: the extraordinary plasticity of the adipose organ. *Eur J Endocrinol* 2014; **170**: R159-R171 [PMID: 24468979

DOI: 10.1530/EJE-13-0945]
89 **Boguslavsky S**, Chiu T, Foley KP, Osorio-Fuentealba C, Antonescu CN, Bayer KU, Bilan PJ, Klip A. Myo1c binding to submembrane actin mediates insulin-induced tethering of GLUT4 vesicles. *Mol Biol Cell* 2012; **23**: 4065-4078 [PMID: 22918957 DOI: 10.1091/mbc.E12-04-0263]
90 **Kawaguchi T**, Tamori Y, Yoshikawa M, Kanda H, Kasuga M. Insulin-stimulated fusion of GLUT4 vesicles to plasma membrane is dependent on wortmannin-sensitive insulin signaling pathway in 3T3-L1 adipocytes. *Kobe J Med Sci* 2008; **54**: E209-E216 [PMID: 19258741]
91 **Hwang JT**, Kim SH, Lee MS, Kim SH, Yang HJ, Kim MJ, Kim HS, Ha J, Kim MS, Kwon DY. Anti-obesity effects of ginsenoside Rh2 are associated with the activation of AMPK signaling pathway in 3T3-L1 adipocyte. *Biochem Biophys Res Commun* 2007; **364**: 1002-1008 [PMID: 17971295 DOI: 10.1016/j.bbrc.2007.10.125]
92 **Kanzaki M**, Pessin JE. Insulin-stimulated GLUT4 translocation in adipocytes is dependent upon cortical actin remodeling. *J Biol Chem* 2001; **276**: 42436-42444 [PMID: 11546823 DOI: 10.1074/jbc.M108297200]
93 **Dissanayake WC**, Sorrenson B, Cognard E, Hughes WE, Shepherd PR. β-catenin is important for the development of an insulin responsive pool of GLUT4 glucose transporters in 3T3-L1 adipocytes. *Exp Cell Res* 2018; **366**: 49-54 [PMID: 29540328 DOI: 10.1016/j.yexcr.2018.03.011]
94 **Li DT**, Habtemichael EN, Julca O, Sales CI, Westergaard XO, DeVries SG, Ruiz D, Sayal B, Bogan JS. Focus: Organelles: GLUT4 Storage Vesicles: Specialized Organelles for Regulated Trafficking. *The Yale J Biol Med* 2019; **92**: 453
95 **Tzeng YM**, Chen K, Rao YK, Lee MJ. Kaempferitrin activates the insulin signaling pathway and stimulates secretion of adiponectin in 3T3-L1 adipocytes. *Eur J Pharmacol* 2009; **607**: 27-34 [PMID: 19326566 DOI: 10.1016/j.ejphar.2009.01.023]
96 **Ku HC**, Tsuei YW, Kao CC, Weng JT, Shih LJ, Chang HH, Liu CW, Tsai SW, Kuo YC, Kao YH. Green tea (-)-epigallocatechin gallate suppresses IGF-I and IGF-II stimulation of 3T3-L1 adipocyte glucose uptake *via* the glucose transporter 4, but not glucose transporter 1 pathway. *Gen Comp Endocrinol* 2014; **199**: 46-55 [PMID: 24486085 DOI: 10.1016/j.ygcen.2014.01.008]
97 **Park SY**, Lee JH, Kim KY, Kim EK, Yun SJ, Kim CD, Lee WS, Hong KW. Cilostazol increases 3T3-L1 preadipocyte differentiation with improved glucose uptake associated with activation of peroxisome proliferator-activated receptor-gamma transcription. *Atherosclerosis* 2008; **201**: 258-265 [PMID: 18355828 DOI: 10.1016/j.atherosclerosis.2008.02.006]
98 **Carlson CJ**, Rondinone CM. Pharmacological inhibition of p38 MAP kinase results in improved glucose uptake in insulin-resistant 3T3-L1 adipocytes. *Metabolism* 2005; **54**: 895-901 [PMID: 15988698 DOI: 10.1016/j.metabol.2005.02.003]
99 **Fatima LA**, Campello RS, Barreto-Andrade JN, Passarelli M, Santos RS, Clegg DJ, Machado UF. Estradiol stimulates adipogenesis and Slc2a4/GLUT4 expression *via* ESR1-mediated activation of CEBPA. *Mol Cell Endocrinol* 2019; **498**: 110447 [PMID: 31100494 DOI: 10.1016/j.mce.2019.05.006]
100 **Bhasker S**, Madhav H, Chinnamma M. Molecular evidence of insulinomimetic property exhibited by steviol and stevioside in diabetes induced L6 and 3T3L1 cells. *Phytomedicine* 2015; **22**: 1037-1044 [PMID: 26407946 DOI: 10.1016/j.phymed.2015.07.007]
101 **Ooi J**, Azmi NH, Imam MU, Alitheen NB, Ismail M. Curculigoside and polyphenol-rich ethyl acetate fraction of Molineria latifolia rhizome improved glucose uptake *via* potential mTOR/AKT activated GLUT4 translocation. *J Food Drug Anal* 2018; **26**: 1253-1264 [PMID: 30249324 DOI: 10.1016/j.jfda.2018.03.003]
102 **Nishina A**, Ukiya M, Fukatsu M, Koketsu M, Ninomiya M, Sato D, Yamamoto J, Kobayashi-Hattori K, Okubo T, Tokuoka H, Kimura H. Effects of Various 5,7-Dihydroxyflavone Analogs on Adipogenesis in 3T3-L1 Cells. *Biol Pharm Bull* 2015; **38**: 1794-1800 [PMID: 26521830 DOI: 10.1248/bpb.b15-00489]
103 **Yin J**, Zuberi A, Gao Z, Liu D, Liu Z, Ye J. Shilianhua extract inhibits GSK-3beta and promotes glucose metabolism. *Am J Physiol Endocrinol Metab* 2009; **296**: E1275-E1280 [PMID: 19351808 DOI: 10.1152/ajpendo.00092.2009]
104 **Lee YS**, Cha BY, Choi SS, Harada Y, Choi BK, Yonezawa T, Teruya T, Nagai K, Woo JT. Fargesin improves lipid and glucose metabolism in 3T3-L1 adipocytes and high-fat diet-induced obese mice. *Biofactors* 2012; **38**: 300-308 [PMID: 22674784 DOI: 10.1002/biof.1022]
105 **Xu X**, Saadeldeen FSA, Xu L, Zhao Y, Wei J, Wang HD, Liu Z, Kang W. The Mechanism of Phillyrin from the Leaves of *Forsythia suspensa* for Improving Insulin Resistance. *Biomed Res Int* 2019; **2019**: 3176483 [PMID: 31355254 DOI: 10.1155/2019/3176483]
106 **Chen L**, Li QY, Shi XJ, Mao SL, Du YL. 6-Hydroxydaidzein enhances adipocyte differentiation and glucose uptake in 3T3-L1 cells. *J Agric Food Chem* 2013; **61**: 10714-10719 [PMID: 24180341 DOI: 10.1021/jf402694m]
107 **Zhou X**, Wang F, Yang H, Chen J, Ren Y, Yuan Z, Wang X, Wang Y. Seleniumenriched exopolysaccharides produced by Enterobacter cloacae Z0206 alleviate adipose inflammation in diabetic KKAy mice through the AMPK/SirT1 pathway. *Mol Med Rep* 2014; **9**: 683-688 [PMID: 24337047 DOI: 10.3892/mmr.2013.1859]
108 **Mazibuko SE**, Joubert E, Johnson R, Louw J, Opoku AR, Muller CJ. Aspalathin improves glucose and lipid metabolism in 3T3-L1 adipocytes exposed to palmitate. *Mol Nutr Food Res* 2015; **59**: 2199-2208 [PMID: 26310822 DOI: 10.1002/mnfr.201500258]
109 **Prasad CN**, Anjana T, Banerji A, Gopalakrishnapillai A. Gallic acid induces GLUT4 translocation and glucose uptake activity in 3T3-L1 cells. *FEBS Lett* 2010; **584**: 531-536 [PMID: 19962377 DOI: 10.1016/j.febslet.2009.11.092]
110 **Gao Y**, Yang MF, Su YP, Jiang HM, You XJ, Yang YJ, Zhang HL. Ginsenoside Re reduces insulin resistance through activation of PPAR-γ pathway and inhibition of TNF-α production. *J Ethnopharmacol* 2013; **147**: 509-516 [PMID: 23545455 DOI: 10.1016/j.jep.2013.03.057]
111 **Schreiber I**, Dörpholz G, Ott CE, Kragesteen B, Schanze N, Lee CT, Köhrle J, Mundlos S, Ruschke K, Knaus P. BMPs as new insulin sensitizers: enhanced glucose uptake in mature 3T3-L1 adipocytes *via* PPARγ and GLUT4 upregulation. *Sci Rep* 2017; **7**: 17192 [PMID: 29222456 DOI: 10.1038/s41598-017-17595-5]

112 **Jackson RM**, Griesel BA, Gurley JM, Szweda LI, Olson AL. Glucose availability controls adipogenesis in mouse 3T3-L1 adipocytes *via* up-regulation of nicotinamide metabolism. *J Biol Chem* 2017; **292**: 18556-18564 [PMID: 28916720 DOI: 10.1074/jbc.M117.791970]

113 **Furuya DT**, Poletto AC, Freitas HS, Machado UF. Inhibition of cannabinoid CB1 receptor upregulates Slc2a4 expression *via* nuclear factor-κB and sterol regulatory element-binding protein-1 in adipocytes. *J Mol Endocrinol* 2012; **49**: 97-106 [PMID: 22735681 DOI: 10.1530/JME-12-0037]

114 **Hesse D**, Hommel A, Jaschke A, Moser M, Bernhardt U, Zahn C, Kluge R, Wittschen P, Gruber AD, Al-Hasani H, Joost HG, Schürmann A. Altered GLUT4 trafficking in adipocytes in the absence of the GTPase Arfrp1. *Biochem Biophys Res Commun* 2010; **394**: 896-903 [PMID: 20230794 DOI: 10.1016/j.bbrc.2010.03.059]

115 **Song H**, Ding L, Zhang S, Wang W. MiR-29 family members interact with SPARC to regulate glucose metabolism. *Biochem Biophys Res Commun* 2018; **497**: 667-674 [PMID: 29462611 DOI: 10.1016/j.bbrc.2018.02.129]

116 **Cooke DW**, Patel YM. GLUT4 expression in 3T3-L1 adipocytes is repressed by proteasome inhibition, but not by inhibition of calpains. *Mol Cell Endocrinol* 2005; **232**: 37-45 [PMID: 15737467 DOI: 10.1016/j.mce.2004.12.008]

117 **Shi Z**, Zhao C, Guo X, Ding H, Cui Y, Shen R, Liu J. Differential expression of microRNAs in omental adipose tissue from gestational diabetes mellitus subjects reveals miR-222 as a regulator of ERα expression in estrogen-induced insulin resistance. *Endocrinology* 2014; **155**: 1982-1990 [PMID: 24601884 DOI: 10.1210/en.2013-2046]

118 **Pessler-Cohen D**, Pekala PH, Kovsan J, Bloch-Damti A, Rudich A, Bashan N. GLUT4 repression in response to oxidative stress is associated with reciprocal alterations in C/EBP alpha and delta isoforms in 3T3-L1 adipocytes. *Arch Physiol Biochem* 2006; **112**: 3-12 [PMID: 16754198 DOI: 10.1080/13813450500500399]

119 **Tryggestad JB**, Teague AM, Sparling DP, Jiang S, Chernausek SD. Macrophage-Derived microRNA-155 Increases in Obesity and Influences Adipocyte Metabolism by Targeting Peroxisome Proliferator-Activated Receptor Gamma. *Obesity (Silver Spring)* 2019; **27**: 1856-1864 [PMID: 31531958 DOI: 10.1002/oby.22616]

120 **Sano H**, Peck GR, Kettenbach AN, Gerber SA, Lienhard GE. Insulin-stimulated GLUT4 protein translocation in adipocytes requires the Rab10 guanine nucleotide exchange factor Dennd4C. *J Biol Chem* 2011; **286**: 16541-16545 [PMID: 21454697 DOI: 10.1074/jbc.C111.228908]

121 **Brewer PD**, Romenskaia I, Kanow MA, Mastick CC. Loss of AS160 Akt substrate causes Glut4 protein to accumulate in compartments that are primed for fusion in basal adipocytes. *J Biol Chem* 2011; **286**: 26287-26297 [PMID: 21613213 DOI: 10.1074/jbc.M111.253880]

122 **Ren W**, Cheema S, Du K. The association of ClipR-59 protein with AS160 modulates AS160 protein phosphorylation and adipocyte Glut4 protein membrane translocation. *J Biol Chem* 2012; **287**: 26890-26900 [PMID: 22689584 DOI: 10.1074/jbc.M112.357699]

123 **Balamatsias D**, Kong AM, Waters JE, Sriratana A, Gurung R, Bailey CG, Rasko JE, Tiganis T, Macaulay SL, Mitchell CA. Identification of P-Rex1 as a novel Rac1-guanine nucleotide exchange factor (GEF) that promotes actin remodeling and GLUT4 protein trafficking in adipocytes. *J Biol Chem* 2011; **286**: 43229-43240 [PMID: 22002247 DOI: 10.1074/jbc.M111.306621]

124 **Yamaguchi S**, Katahira H, Ozawa S, Nakamichi Y, Tanaka T, Shimoyama T, Takahashi K, Yoshimoto K, Imaizumi MO, Nagamatsu S, Ishida H. Activators of AMP-activated protein kinase enhance GLUT4 translocation and its glucose transport activity in 3T3-L1 adipocytes. *Am J Physiol Endocrinol Metab* 2005; **289**: E643-E649 [PMID: 15928020 DOI: 10.1152/ajpendo.00456.2004]

125 **Berg J**, Tymoczko J, Berg JM TJ, Stryer L. Biochemistry. 5th ed. New York: WH freeman, section. Section 30.2 Each Organ Has a Unique Metabolic Profile, 2002: 18

126 **Santalucía T**, Camps M, Castelló A, Muñoz P, Nuel A, Testar X, Palacin M, Zorzano A. Developmental regulation of GLUT-1 (erythroid/Hep G2) and GLUT-4 (muscle/fat) glucose transporter expression in rat heart, skeletal muscle, and brown adipose tissue. *Endocrinology* 1992; **130**: 837-846 [PMID: 1370797 DOI: 10.1210/endo.130.2.1370797]

127 **Mooradian AD**, Chehade JM, Kim J. Age-related changes in thyroid hormone effects on glucose transporter isoforms of rat heart. *Life Sci* 1999; **65**: 981-989 [PMID: 10499866 DOI: 10.1016/s0024-3205(99)00342-2]

128 **Martineau LC**, Chadan SG, Parkhouse WS. Age-associated alterations in cardiac and skeletal muscle glucose transporters, insulin and IGF-1 receptors, and PI3-kinase protein contents in the C57BL/6 mouse. *Mech Ageing Dev* 1999; **106**: 217-232 [PMID: 10100151 DOI: 10.1016/s0047-6374(98)00106-7]

129 **Wang F**, Liang GY, Liu DX, Tang Q, Zhang J, Cai QY, Zhang DS, Han X. Effect of Si-RNA-silenced HIF-1α gene on myocardial ischemia-reperfusion-induced insulin resistance. *Int J Clin Exp Med* 2015; **8**: 15514-15520 [PMID: 26629042]

130 **Santalucía T**, Moreno H, Palacín M, Yacoub MH, Brand NJ, Zorzano A. A novel functional co-operation between MyoD, MEF2 and TRalpha1 is sufficient for the induction of GLUT4 gene transcription. *J Mol Biol* 2001; **314**: 195-204 [PMID: 11718554 DOI: 10.1006/jmbi.2001.5091]

131 **Michael LF**, Wu Z, Cheatham RB, Puigserver P, Adelmant G, Lehman JJ, Kelly DP, Spiegelman BM. Restoration of insulin-sensitive glucose transporter (GLUT4) gene expression in muscle cells by the transcriptional coactivator PGC-1. *Proc Natl Acad Sci USA* 2001; **98**: 3820-3825 [PMID: 11274399 DOI: 10.1073/pnas.061035098]

132 **Maria Z**, Campolo AR, Lacombe VA. Diabetes Alters the Expression and Translocation of the Insulin-Sensitive Glucose Transporters 4 and 8 in the Atria. *PLoS One* 2015; **10**: e0146033 [PMID: 26720696 DOI: 10.1371/journal.pone.0146033]

133 **Dutka DP**, Pitt M, Pagano D, Mongillo M, Gathercole D, Bonser RS, Camici PG. Myocardial glucose transport and utilization in patients with type 2 diabetes mellitus, left ventricular dysfunction, and coronary artery disease. *J Am Coll Cardiol* 2006; **48**: 2225-2231 [PMID: 17161251 DOI: 10.1016/j.jacc.2006.06.078]

134 **Zheng X**, Wang S, Zou X, Jing Y, Yang R, Li S, Wang F. Ginsenoside Rb1 improves cardiac function and

remodeling in heart failure. *Exp Anim* 2017; **66**: 217-228 [PMID: 28367863 DOI: 10.1538/expanim.16-0121]

135 **Chen L**, Wang F, Sun X, Zhou J, Gao L, Jiao Y, Hou X, Qin CY, Zhao J. Chronic ethanol feeding impairs AMPK and MEF2 expression and is associated with GLUT4 decrease in rat myocardium. *Exp Mol Med* 2010; **42**: 205-215 [PMID: 20164678 DOI: 10.3858/emm.2010.42.3.021]

136 **Shoop S**, Maria Z, Campolo A, Rashdan N, Martin D, Lovern P, Lacombe VA. Glial Growth Factor 2 Regulates Glucose Transport in Healthy Cardiac Myocytes and During Myocardial Infarction *via* an Akt-Dependent Pathway. *Front Physiol* 2019; **10**: 189 [PMID: 30971932 DOI: 10.3389/fphys.2019.00189]

137 **Gallo MP**, Femminò S, Antoniotti S, Querio G, Alloatti G, Levi R. Catestatin Induces Glucose Uptake and GLUT4 Trafficking in Adult Rat Cardiomyocytes. *Biomed Res Int* 2018; **2018**: 2086109 [PMID: 30370303 DOI: 10.1155/2018/2086109]

138 **Webster I**, Friedrich SO, Lochner A, Huisamen B. AMP kinase activation and glut4 translocation in isolated cardiomyocytes. *Cardiovasc J Afr* 2010; **21**: 72-78 [PMID: 20532430]

139 **Soltésová D**, Veselá A, Mravec B, Herichová I. Daily profile of glut1 and glut4 expression in tissues inside and outside the blood-brain barrier in control and streptozotocin-treated rats. *Physiol Res* 2013; **62** Suppl 1: S115-S124 [PMID: 24329691]

140 **Yuan Y**, Liu X, Miao H, Huang B, Liu Z, Chen J, Quan X, Zhu L, Dong H, Zhang Z. PEDF increases GLUT4-mediated glucose uptake in rat ischemic myocardium *via* PI3K/AKT pathway in a PEDFR-dependent manner. *Int J Cardiol* 2019; **283**: 136-143 [PMID: 30819588 DOI: 10.1016/j.ijcard.2019.02.035]

141 **Giannocco G**, Oliveira KC, Crajoinas RO, Venturini G, Salles TA, Fonseca-Alaniz MH, Maciel RM, Girardi AC. Dipeptidyl peptidase IV inhibition upregulates GLUT4 translocation and expression in heart and skeletal muscle of spontaneously hypertensive rats. *Eur J Pharmacol* 2013; **698**: 74-86 [PMID: 23051671 DOI: 10.1016/j.ejphar.2012.09.043]

142 **Sertié RA**, Sertié AL, Giannocco G, Poyares LL, Nunes MT. Acute growth hormone administration increases myoglobin expression and Glut4 translocation in rat cardiac muscle cells. *Metabolism* 2014; **63**: 1499-1502 [PMID: 25306099 DOI: 10.1016/j.metabol.2014.08.012]

143 143 Standring S. Gray's anatomy: the anatomical basis of clinical practice. Standring S, Gray H, editors. Churchill Livingstone Elsevier, 2015

144 **McNay EC**, Williamson A, McCrimmon RJ, Sherwin RS. Cognitive and neural hippocampal effects of long-term moderate recurrent hypoglycemia. *Diabetes* 2006; **55**: 1088-1095 [PMID: 16567533 DOI: 10.2337/diabetes.55.04.06.db05-1314]

145 **Pearson-Leary J**, McNay EC. Intrahippocampal administration of amyloid-β(1-42) oligomers acutely impairs spatial working memory, insulin signaling, and hippocampal metabolism. *J Alzheimers Dis* 2012; **30**: 413-422 [PMID: 22430529 DOI: 10.3233/jad-2012-112192]

146 **Weinger K**, Jacobson AM, Draelos MT, Finkelstein DM, Simonson DC. Blood glucose estimation and symptoms during hyperglycemia and hypoglycemia in patients with insulin-dependent diabetes mellitus. *Am J Med* 1995; **98**: 22-31 [PMID: 7825615 DOI: 10.1016/s0002-9343(99)80077-1]

147 **Shin J**, Tsui W, Li Y, Lee SY, Kim SJ, Cho SJ, Kim YB, de Leon MJ. Resting-state glucose metabolism level is associated with the regional pattern of amyloid pathology in Alzheimer's disease. *Int J Alzheimers Dis* 2011; **2011**: 759780 [PMID: 21461406 DOI: 10.4061/2011/759780]

148 **Biessels GJ**, Reagan LP. Hippocampal insulin resistance and cognitive dysfunction. *Nat Rev Neurosci* 2015; **16**: 660-671 [PMID: 26462756 DOI: 10.1038/nrn4019]

149 **Hirvonen J**, Virtanen KA, Nummenmaa L, Hannukainen JC, Honka MJ, Bucci M, Nesterov SV, Parkkola R, Rinne J, Iozzo P, Nuutila P. Effects of insulin on brain glucose metabolism in impaired glucose tolerance. *Diabetes* 2011; **60**: 443-447 [PMID: 21270256 DOI: 10.2337/db10-0940]

150 **Anand KS**, Dhikav V. Hippocampus in health and disease: An overview. *Ann Indian Acad Neurol* 2012; **15**: 239-246 [PMID: 23349586 DOI: 10.4103/0972-2327.104323]

151 **McNay EC**, Ong CT, McCrimmon RJ, Cresswell J, Bogan JS, Sherwin RS. Hippocampal memory processes are modulated by insulin and high-fat-induced insulin resistance. *Neurobiol Learn Mem* 2010; **93**: 546-553 [PMID: 20176121 DOI: 10.1016/j.nlm.2010.02.002]

152 **Kern W**, Peters A, Fruehwald-Schultes B, Deininger E, Born J, Fehm HL. Improving influence of insulin on cognitive functions in humans. *Neuroendocrinology* 2001; **74**: 270-280 [PMID: 11598383 DOI: 10.1159/000054694]

153 **Craft S**, Newcomer J, Kanne S, Dagogo-Jack S, Cryer P, Sheline Y, Luby J, Dagogo-Jack A, Alderson A. Memory improvement following induced hyperinsulinemia in Alzheimer's disease. *Neurobiol Aging* 1996; **17**: 123-130 [PMID: 8786794 DOI: 10.1016/0197-4580(95)02002-0]

154 **Reno CM**, Puente EC, Sheng Z, Daphna-Iken D, Bree AJ, Routh VH, Kahn BB, Fisher SJ. Brain GLUT4 Knockout Mice Have Impaired Glucose Tolerance, Decreased Insulin Sensitivity, and Impaired Hypoglycemic Counterregulation. *Diabetes* 2017; **66**: 587-597 [PMID: 27797912 DOI: 10.2337/db16-0917]

155 **Bakirtzi K**, Belfort G, Lopez-Coviella I, Kuruppu D, Cao L, Abel ED, Brownell AL, Kandror KV. Cerebellar neurons possess a vesicular compartment structurally and functionally similar to Glut4-storage vesicles from peripheral insulin-sensitive tissues. *J Neurosci* 2009; **29**: 5193-5201 [PMID: 19386915 DOI: 10.1523/jneurosci.0858-09.2009]

156 **Agrawal R**, Vieira-de-Abreu A, Durupt G, Taylor C, Chan O, Fisher SJ. Insulin regulates GLUT4 in the ventromedial hypothalamus to restore the sympathoadrenal response to hypoglycemia in diabetic rats. *Am J Physiol Endocrinol Metab* 2018; **315**: E1286-E1295 [PMID: 30226996 DOI: 10.1152/ajpendo.00324.2018]

157 **Ashrafi G**, Wu Z, Farrell RJ, Ryan TA. GLUT4 Mobilization Supports Energetic Demands of Active Synapses. *Neuron* 2017; **93**: 606-615. e3 [PMID: 28111082 DOI: 10.1016/j.neuron.2016.12.020]

158 **El Messari S**, Aït-Ikhlef A, Ambroise DH, Penicaud L, Arluison M. Expression of insulin-responsive glucose transporter GLUT4 mRNA in the rat brain and spinal cord: an in situ hybridization study. *J Chem Neuroanat* 2002; **24**: 225-242 [PMID: 12406499 DOI: 10.1016/s0891-0618(02)00058-3]

159 **Funk N**, Munz M, Ott T, Brockmann K, Wenninger-Weinzierl A, Kühn R, Vogt-Weisenhorn D, Giesert F,

Wurst W, Gasser T, Biskup S. The Parkinson's disease-linked Leucine-rich repeat kinase 2 (LRRK2) is required for insulin-stimulated translocation of GLUT4. *Sci Rep* 2019; **9**: 4515 [PMID: 30872638 DOI: 10.1038/s41598-019-40808-y]

160 **Ismail MA**, Mateos L, Maioli S, Merino-Serrais P, Ali Z, Lodeiro M, Westman E, Leitersdorf E, Gulyás B, Olof-Wahlund L, Winblad B, Savitcheva I, Björkhem I, Cedazo-Mínguez A. 27-Hydroxycholesterol impairs neuronal glucose uptake through an IRAP/GLUT4 system dysregulation. *J Exp Med* 2017; **214**: 699-717 [PMID: 28213512 DOI: 10.1084/jem.20160534]

161 **Ngarmukos C**, Baur EL, Kumagai AK. Co-localization of GLUT1 and GLUT4 in the blood-brain barrier of the rat ventromedial hypothalamus. *Brain Res* 2001; **900**: 1-8 [PMID: 11325341 DOI: 10.1016/s0006-8993(01)02184-9]

162 **Ren H**, Vieira-de-Abreu A, Yan S, Reilly AM, Chan O, Accili D. Altered Central Nutrient Sensing in Male Mice Lacking Insulin Receptors in Glut4-Expressing Neurons. *Endocrinology* 2019; **160**: 2038-2048 [PMID: 31199472 DOI: 10.1210/en.2019-00341]

163 **Sankar R**, Thamotharan S, Shin D, Moley KH, Devaskar SU. Insulin-responsive glucose transporters-GLUT8 and GLUT4 are expressed in the developing mammalian brain. *Brain Res Mol Brain Res* 2002; **107**: 157-165 [PMID: 12425944 DOI: 10.1016/s0169-328x(02)00487-4]

World Journal of Biological Chemistry

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

*World J Biol Chem* 2020 November 27; 11(3): 99-111

DOI: 10.4331/wjbc.v11.i3.99

ISSN 1949-8454 (online)

*MINIREVIEWS*

# Deciphering the modifiers for phenotypic variability of X-linked adrenoleukodystrophy

Shruti V Palakuzhiyil, Rita Christopher, Sadanandavalli Retnaswami Chandra

**ORCID number:** Shruti V Palakuzhiyil 0000-0002-3273-4282; Rita Christopher 0000-0002-0024-4748; Sadanandavalli Retnaswami Chandra 0000-0001-9868-9974.

**Author contributions:** All the authors have contributed equally to the preparation of the manuscript.

**Supported by** Department of Biotechnology, New Delhi, India. No. BT/PR26150/MED/12/768/2017.

**Conflict-of-interest statement:** The authors declare no conflicts of interest for this article.



**Manuscript source:** Invited manuscript

**Shruti V Palakuzhiyil, Rita Christopher,** Department of Neurochemistry, National Institute of Mental Health and Neuro Sciences, Bengaluru 560029, India

**Sadanandavalli Retnaswami Chandra,** Department of Neurology, Sri Ramakrishna Ashram Charitable Hospital, Trivandrum 695010, India

**Corresponding author:** Rita Christopher, MD, Professor, Department of Neurochemistry, National Institute of Mental Health and Neuro Sciences, Hosur Road, Bengaluru 560029, India. rita.nimhans@yahoo.com

## Abstract

X-linked adrenoleukodystrophy (X-ALD), an inborn error of peroxisomal β-oxidation, is caused by defects in the ATP Binding Cassette Subfamily D Member 1 (*ABCD1*) gene. X-ALD patients may be asymptomatic or present with several clinical phenotypes varying from severe to mild, severe cerebral adrenoleukodystrophy to mild adrenomyeloneuropathy (AMN). Although most female heterozygotes present with AMN-like symptoms after 60 years of age, occasional cases of females with the cerebral form have been reported. Phenotypic variability has been described within the same kindreds and even among monozygotic twins. There is no association between the nature of *ABCD1* mutation and the clinical phenotypes, and the molecular basis of phenotypic variability in X-ALD is yet to be resolved. Various genetic, epigenetic, and environmental influences are speculated to modify the disease onset and severity. In this review, we summarize the observations made in various studies investigating the potential modifying factors regulating the clinical manifestation of X-ALD, which could help understand the pathogenesis of the disease and develop suitable therapeutic strategies.

**Key Words:** X-adrenoleukodystrophy; Cerebral adrenoleukodystrophy; Adrenomyeloneuropathy; Phenotypic variation; Modifiers



**Core Tip:** The monogenic peroxisomal disorder, X-linked adrenoleukodystrophy (X-

**Specialty type:** Neurosciences

**Country/Territory of origin:** India

**Peer-review report's scientific quality classification**

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

**Received:** June 4, 2020
**Peer-review started:** June 4, 2020
**First decision:** July 4, 2020
**Revised:** July 10, 2020
**Accepted:** September 18, 2020
**Article in press:** September 18, 2020
**Published online:** November 27, 2020

**P-Reviewer:** Demarquoy J
**S-Editor:** Wang JL
**L-Editor:** Filipodia
**P-Editor:** Li X

ALD), presents with different clinical phenotypes. The molecular basis for the phenotypic variation has yet to be resolved and is considered to be influenced by genetic, epigenetic, cellular, or environmental factors. We herein discuss the various modifying factors, which can potentially alter the phenotypic presentation of X-ALD.

**Citation:** Palakuzhiyil SV, Christopher R, Chandra SR. Deciphering the modifiers for phenotypic variability of X-linked adrenoleukodystrophy. *World J Biol Chem* 2020; 11(3): 99-111
**URL:** https://www.wjgnet.com/1949-8454/full/v11/i3/99.htm
**DOI:** https://dx.doi.org/10.4331/wjbc.v11.i3.99

## INTRODUCTION

Monogenic disorders are primarily caused by a single defective gene, but mutations in a single gene can result in a disease with varying clinical phenotypes. X-linked adrenoleukodystrophy (X-ALD), caused by mutations in the *ABCD1* gene, is one such monogenic disorder affecting peroxisomal β-oxidation. *ABCD1,* mapped on Xq28, comprises of 10 exons[1] and codes for a 75kDa peroxisomal membrane protein called the ABCD1 protein or adrenoleukodystrophy protein (ALDP)[2]. ALDP is highly expressed in specific cell types like oligodendrocytes, astrocytes, microglial cells, adrenocortical cells, and endothelial cells in the brain, adrenal glands, testis, and kidney, liver, lung, and placenta[1,3,4]. ALDP transports very-long-chain fatty acids (VLCFAs), activated by coenzyme-A, into the peroxisomes, for β oxidation. A defect in the *ABCD1* gene results in the synthesis of a dysfunctional ALDP protein, unable to transport VLCFA across the peroxisomal membrane. This leads to the buildup of VLCFA, mainly hexacosanoic and tetracosanoic acids, in various body tissues, primarily the brain, spinal cord, adrenal cortex, testis, and plasma[1]. The elevated plasma concentration of VLCFA acts as a diagnostic marker for this disorder.

The exact role of VLCFA in the pathogenesis of X-ALD remains unclear, and no correlation has been established between the concentration of VLCFA and the different phenotypes of X-ALD. The abnormally accumulated VLCFA can disrupt the integrity of the plasma membranes through interdigitating between the leaflets of the lipid bilayer and can induce lipotoxicity, endoplasmic reticulum stress, mitochondrial dysfunction, and oxidative stress leading to apoptosis favoring the process of cerebral demyelination in the brain[5-8].

## CLINICAL SPECTRUM OF X-ALD

X-ALD patients have a diverse clinical presentation. They may be asymptomatic or present with the rapidly progressive forms after 3 years of age[1]. The main types of presentation in male patients are: (1) Cerebral ALD (CALD), the cerebral demyelinating form; (2) Adrenomyeloneuropathy (AMN), with spinal cord demyelination and axonal degeneration; and (3) Addison-like phenotype due to adrenocortical insufficiency.

Cerebral ALD affects males and is typified by progressive inflammatory cerebral demyelination leading to neurodegeneration. It includes the childhood cerebral form (CCALD) - appearing in mid-childhood (4-8 years), adolescent cerebral form or adolescent CALD (10-20 years), and adult cerebral form or adult CALD (> 20 years). Children with CCALD present with behavioral problems and a decline in school performance due to impairment of auditory discrimination and spatial orientation, thereby affecting writing and speech. Rarely, seizures may be the initial manifestation. As the disease progresses, there are further signs of damage to the brain white matter, including spastic quadriparesis, dysphagia, and visual loss leading to a vegetative condition. Adolescent CALD manifests between 10 and 20 years of age with clinical features of cerebral involvement. In adult CALD, psychiatric symptoms, seizures, spastic paraparesis, and dementia develop in males over the age of 20.

The second most common phenotype, AMN, is usually characterized as a gradually developing, non-inflammatory axonopathy, mainly affecting males over 20 years of age. AMN is sub-divided as "pure AMN" and "AMN-cerebral." In patients with pure

AMN, there is spinal cord involvement resulting in gait disturbances and bladder dysfunction, whereas patients with AMN-cerebral form show clinical features of cerebral inflammation besides the symptoms of pure AMN[9]. The transformation of pure AMN to the cerebral form of AMN is not clearly understood.

A significant proportion of male patients with X-ALD develop adrenocortical insufficiency, which may occur either after the appearance of neurological symptoms or decades ahead. A majority of males present with adrenocortical insufficiency in association with features of CALD or AMN[1]. Rare cases have shown to manifest adrenocortical insufficiency without cerebral demyelination and are characterized as "Addison only" type of X-ALD[1,10].

In 20%-50% of heterozygotes or female carriers of X-ALD, symptoms similar to AMN, typically consisting of gait disturbance, dysuria, and urgency, occur after 40 years. There are also reports of female carriers with CALD and adrenal insufficiency[11]. For instance, Hershkovitz *et al*[12] reported a case of CALD in a girl of age, 8.9 years, where the genetic analysis was indicative of heterozygosity with a deletion at Xq27.2-tel. Similarly, Chen *et al*[13] reported CALD and adrenal insufficiency in a 38-year-old Chinese woman. The possible explanation for the symptomatic state observed in certain heterozygotes could be skewed X-chromosome inactivation, resulting in the expression of the chromosome carrying the faulty *ABCD*1 gene[14]. Studies have found a significant association between the degree of skewness and the severity of neurological deficits[14,15]. However, factors favoring this event remain unidentified.

## PHENOTYPIC VARIABILITY IN THE SAME PEDIGREE

The various clinical types of X-ALD frequently appear in the same kindreds and nuclear families carrying the same mutation in the *ABCD*1 gene. In half of the kindreds, both CALD and AMN are found[16]. Diverse phenotypes and clinical features have been seen in mother and son, monozygotic twins, heterozygous siblings, and affected members of several generations of families[17-19]. For instance, a study reported a family with X-ALD where the proband was diagnosed with the CCALD. In contrast, his two other siblings and maternal uncle were diagnosed with the adolescent form of CALD, Addison's only phenotype of X-ALD, and AMN. Mutational analysis found a hemizygous mutation of *c.1780C>G* in the *ABCD1* gene in all three siblings[20].

A Brazilian study reports two siblings with CCALD presenting with different clinical features at diagnosis. Both parents had the p.Trp132Ter mutation in *ABCD*1. Addison's disease phenotype was found in their maternal grandfather[21]. Similarly, different clinical phenotypes have been reported in a Tunisian family with p.Gln316Pro mutation in *ABCD1*[22].

In an early study of 15 Dutch kindreds, van Geel and coworkers[23] found only CALD in 20%, only AMN in 40%, and both CALD and AMN in 40%. Another large study of 178 kindreds found CALD in 30%, AMN in 20%, and both CALD and AMN in 50%[1].

Korenke *et al*[19] describe phenotypic variation in monozygotic twins with the same mutation (C2203T) in exon8 of *ABCD1*, where neuroimaging studies were found normal for the first twin, and parietooccipital demyelination was found in the second twin at ten years of age. Sobue *et al*[24] also report genetically confirmed monozygotic twins who presented with different clinical types of X-ALD. Although myeloneuropathy was present in both twins, widespread brain demyelination with cognitive dysfunction and behavioral symptoms was pronounced in the older twin, while the younger twin presented with adrenal insufficiency.

## CAUSE OF PHENOTYPIC VARIABILITY IN X-ALD

Despite various studies, the exact cause of the phenotypic variability is not clear. Over > 800 mutations have been characterized in the *ABCD*1 gene, but, based on the observations of various studies, it is clear that there is no association between the genotype and the different phenotypes of X-ALD. Identical defects in the *ABCD*1 gene have been found in cases with different types of X-ALD (CCALD, adult CALD, AMN, Addison only, and asymptomatic)[25,26]. Mutations that can cause deleterious damage to the protein, such as large deletions are reported in severe cerebral forms and milder types such as AMN and asymptomatic cases[27]. These data support the assumption that factors other than the X-linked locus participate in deciding the phenotype. It is

possible that the specific mutation, along with the influence of individual genetic and environmental factors commonly referred to as "modifiers", could play a crucial role in penetrance and the disease severity. Exploring these potential modifiers and understanding their roles in defining the phenotype in X-ALD associated with a specific mutation in the *ABCD1* gene is crucial in predicting the disease phenotype.

## POTENTIAL MODIFIERS IN X-ALD

A variant at a particular genetic locus may not be adequate to determine the clinical phenotype, severity, and progression in human diseases[28]. Direct or indirect association of different genetic, epigenetic factors, and environmental factors can change the expressivity, penetrance, and severity of a disease progression (Figure 1). The relative involvement of multiple modifiers to the disease phenotype may generate a combined impact on the phenotypic expression, and the combination of these modifiers may differ among individuals. Identifying these modifying factors and establishing a collective association with different clinical phenotypes is very challenging, but maybe crucial for appropriate management of the disease.

### *Modifier genes*

Phenotypic variability of a disease can be explained by the influence of other genes apart from the gene involved in the disease, and these genes are called "modifier genes"[29]. Modifier genes can affect the expression or function of another gene. The final impact of these genes on clinical variability could depend on their collective interaction and the interplay of other epigenetic or environmental factors. Genetic segregation analysis of a considerable number of families with X-ALD and analysis of concordant and discordant siblings indicates that a modifying gene, with an allele frequency of approximately 0.5, could be the main determining factor for phenotypic differences[16,30]. Numerous studies have been directed towards identifying potential modifier genes that control the clinical variability of X-ALD. The foremost challenge for many studies is the small sample size for detecting the genetic association. These studies have attempted to investigate a modifier role in various genes involved in the metabolism of VLCFA, inflammatory pathways, methionine metabolism, and bile acid metabolism (Table 1). However, no studies have attempted to elucidate an interactive association of different genes with different phenotypes associated with the primary mutation in the *ABCD1* gene.

**Genes involved in peroxisomal metabolism of VLCFA:** The molecular defect in X-ALD is a deficiency of the ALDP protein due to which there is a defective passage of VLCFA into the peroxisome. In the peroxisomal matrix, saturated and unbranched VLCFA are metabolized by enzymes of the β-oxidation pathway[31]. In patients with X-ALD, VLCFA, particularly C26:0, collect in various tissues and are incorporated into different complex lipids. Excess levels of VLCFAs and VLCFA-containing lipids are considered as biochemical triggers playing a central part in the development of X-ALD.

The superfamily of ATP-binding cassette transporters, which ALDP belongs to, also includes ALDRP, PMP70, and ABCD4 coded by *ABCD*2, *ABCD3*, and *ABCD4* genes. Experimental data suggest that *ABCD*2 and *ABCD*3 genes, when over-expressed, can supplement the biochemical defect in ALD fibroblasts[32]. However, Asheuer *et al*[33] demonstrated that the concentrations of *ABCD*2 transcripts were similar in the unaffected white brain matter in different ALD phenotypes suggesting that difference in *ABCD*2-gene expression was not likely to contribute to the vulnerability for cerebral demyelination. In contrast, the expression of *ABCD4* genes correlated with the predisposition for brain demyelination and showed a trend of an association with CCALD, AMN-cerebral, and pure AMN phenotypes. Two other independent association studies have reported that ALD phenotypes are not associated with the *ABCD*2 genotype[34]. A Japanese study found no significant association of SNPs in *ABCD*2, *ABCD3*, and *ABCD4*, and ALD phenotypes, except for five single nucleotide polymorphisms in *ABCD*4, were less commonly found in AMN patients than in controls, but no significant association with CCALD (Table 1). However, a repetition of this study of five SNPs on another group of French ALD patients found no significant link with CCALD or pure AMN[35].

Accumulation of VLCFA could also result from the excessive lengthening of long-chain fatty acids to VLCFA in the cell[36]. This increased elongation can be due to enhanced expression of elongases and/or imbalance in the degradation and synthesis

**Table 1 List of potential modifier genes for X-linked adrenoleukodystrophy investigated in various studies**

| Series No. | Gene name | Variants studied | No. of cases | Inference | Ref. |
|---|---|---|---|---|---|
| **Genes associated with VLCFA metabolism** | | | | | |
| I | *ABCD2* | rs11172566 | 117 | No significance | Maier *et al*[34] |
| | | rs11172661 | | No significance | |
| I | *ABCD2* | A/T(5'UTR) | 280 | No significance | Matsukawa *et al*[35] |
| | | M94V | | No significance | |
| II | *ABCD3* | rs4148058 | 280 | No significance | Matsukawa *et al*[35] |
| | | rs2147794 | | No significance | |
| | | rs16946 | | No significance | |
| | | rs681187 | | No significance | |
| | | rs662813 | | No significance | |
| | | rs337592 | | No significance | |
| III | *ABCD4* | rs17182959 | 280 | No significance | Matsukawa *et al*[35] |
| | | rs17158118 | | No significance | |
| | | rs17782508 | | No significance | |
| | | rs2301345 | | No significance | |
| | | rs4148077 | | No significance | |
| | | rs4148078 | | No significance | |
| | | rs3742801 | | No significance | |
| IV | Cytochrome P450 4F subfamily (*CYP4F2*) | rs21086622 | 152 | Minor allele A associated with CALD ($P$ = 0.036) | van Engen *et al*[38] |
| | | rs3093207 | | No significance | |
| | | rs1272 | | No significance | |
| | | rs3093200 | | No significance | |
| | | rs3093194 | | No significance | |
| | | rs3093166 | | No significance | |
| | | rs4808400 | | No significance | |
| | | rs3093153 | | No significance | |
| | | rs3093135 | | No significance | |
| | | rs3093105 | | No significance | |
| **Genes associated with methionine metabolism** | | | | | |
| I | Cystathionine β-Synthase (*CBS*) | c.844_845ins68 | 86 | Associated with pure AMN | Linnebank *et al*[46] |
| I | Cystathionine β-Synthase (*CBS*) | c.844_845ins68 | 172 | No significance | Semmler *et al*[48] |
| II | Methionine synthase (*MTR*) | c.2756A>G | 86 | No significance | Linnebank *et al*[45] |
| II | Methionine synthase (*MTR*) | c.2756A>G | 172 | No significance | Semmler *et al*[48] |
| III | Methylenetetrahydrofolate reductase (*MTHFR*) | c.677C>T | 86 | No significance | Linnebank *et al*[45] |
| III | Methylenetetrahydrofolate reductase (*MTHFR*) | c.677C>T | 172 | No significance | Semmler *et al*[48] |
| | | c.1298A>C | | No significance | |
| IV | Dihydrofolate reductase (*DHFR*) | c.594+59del19bp | 172 | No significance | Semmler |

| | | | | | |
|---|---|---|---|---|---|
| | | | | | *et al*[48] |
| **V** | 5-Methyltetrahydrofolate-Homocysteine Methyltransferase Reductase (*MTRR*) | c.60A>G | 172 | No significance | Semmler *et al*[48] |
| **VI** | Transcobalamin 2 (TC2) | c. 776C>G | 86 | GG genotype prevalent in AMN with demyelination compared to pure AMN ($P$ = 0.001) | Linnebank *et al*[45] |
| **VI** | Transcobalamin 2 (*TC2*) | c. 776C>G (GG) | 172 | GG genotype associated with demyelination ($P$ = 0.036) | Semmler *et al*[48] |
| **VII** | Reduced folate carrier 1 (*RFC1*) | c.80G>A | 172 | No significance | Semmler *et al*[48] |
| **Genes associated with inflammation** | | | | | |
| **I** | *TNF-a* | G- 308A | 15 | No significance | McGuinness *et al*[64] |
| **II** | Cluster of differentiation (*CD1*) | CD1A-CD1E | 139 | No significance | Barbier *et al*[44] |
| **III** | Human leukocyte antigen (*HLA*) | HLA-DRB1*16 | 29 | HLA-DRB1*16 associated with X-ALD ($P$ < 0.02) | Berger *et al*[40] |
| | | HLA-DRB1*15 | | No significance | |
| **III** | Human leukocyte antigen (*HLA*) | HLA-DBR1* | 70 | No significance | Schmidt *et al*[41] |
| **III** | Human leukocyte antigen (*HLA*) | HLA-DRB1*16 | 106 | No significance | McGuinness *et al*[65] |
| | | HLA-DRB1*15 | | | |
| **IV** | Interleukin 6 (*IL6*) | | 68 | No significance | Schmidt *et al*[41] |
| **V** | Myelin Oligodendrocyte glycoprotein (*MOG*) | (TAAA)n | 68 | 226bp (TAAA)n polymorphism associated with the presence of Anti-MOG antibody. ($P$ < 0.05). | Schmidt *et al*[41] |
| **V** | Myelin oligodendrocyte glycoprotein (*MOG*) | G15A | 44 | No significance | Gomez-Lira *et al*[42] |
| | | CTC 5 repeats | | No significance | |
| | | G511C | | No significance | |
| | | G520A | | No significance | |
| | | 551+68 A→G | | No significance | |
| | | 551+77 C→T | | No significance | |
| **Other genes** | | | | | |
| **I** | Superoxide oxide dismutase (*SOD2*) | rs4880 | | T-allele associated with cerebral involvement in non-CCALD cases | Brose *et al*[49] |
| | | rs2758352 | | No significance | |
| | | rs2842980 | | No significance | |
| | | rs2758329 | | No significance | |
| **II** | Apolipoprotein E (*APOE*) | rs7412 rs429358 | 83 | APOE4 associated with cerebral involvement | Orchard *et al*[52] |
| **III** | Cytochrome P450 family 7 subfamily A member 1 (*CYP2A1*) | rs3824260 (c.-533T>C) | Study carried out on a patient diagnosed with AMN with c.659T>C mutation in ABCD1 gene in patient and mother | CC allele observed in patient whereas CT in mother | Platek *et al*[50] |
| | | rs3808607 (c.-267C>A) | | AA allele observed in patient whereas CA in mother | |
| **IV** | 3 β-hydroxysteroid dehydrogenase type 7 (*HSD3B7*) | rs9938550 (c.748A>G) | | GG allele observed in both | |
| | | rs2305880 (c.1068T>C) | | CC allele observed in patient | |
| **V** | Bile acyl-CoA synthetase (*SLC27A5*) | rs4810274 (c.1668-6T>C) | | CC observed in patient | |

| | | | |
|---|---|---|---|
| **VI** | Aldo-keto reductase family 1 member D1 (*AKR1D1*) | c.-71G>C | GC observed in patient |
| **VII** | Cytochrome P450 Family 27 Subfamily A Member 1 (*CYP27A1*) | rs397795841 (c.-357dupC) | Homozygous mutation in both |

AMN: Adrenomyeloneuropathy; CALD: Cerebral adrenoleukodystrophy; CCALD: Childhood cerebral adrenoleukodystrophy; VLCFA: Very-long-chain fatty acid; X-ALD: X-linked adrenoleukodystrophy.

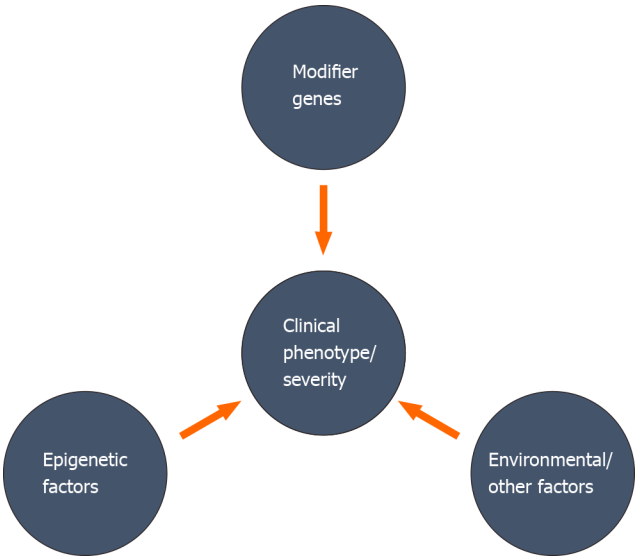


**Figure 1 Possible modifiers associated with disease phenotypic variability.** Phenotypic heterozygosity observed in a monogenic disorder can be due to the association of modifying factors such as genetic, epigenetic, and/or environmental factors, commonly termed as "modifiers."

of VLCFA. Ofman *et al*[37] reported no change in the expression of *ELOVL*1 in X-ALD fibroblast, therefore ruling out the possibility of VLCFA accumulation due to increased expression of *ELOVL1*. However, knockdown of *ELOVL*1 showed a reduction in C26:0 concentrations in X-ALD fibroblasts, thus indicating that *ELOVL*1 could be a possible modifier for X-ALD.

The oxidation of VLCFA starts with its activation by coenzyme A and enzymes with very-long-chain acyl-CoA synthetase (VLACS) activity. In a study of the unaffected brain white matter from X-ALD cases, Asheuer *et al*[33] have found that the expression of VLACS genes did not correlate with the clinical phenotypes (CALD and AMN phenotypes). On the contrary, lower expression of the *BG*1 gene, which codes for a non-peroxisomal synthetase which activates VLCFA to its coenzyme A derivatives, was found in the white matter of ALD patients which correlated with the presence of cerebral demyelination. Hence *BG*1 could be considered as a potential modifier gene[33].

VLCFAs can also undergo ω-oxidation, and are further converted to dicarboxylic acids by the cytochrome P450 system. These reactions may present another route for the metabolism of the accumulated VLCFAs. The gene, *CYP4F2*, codes for a critical enzyme in the ω-oxidation of VLCFA to very long-chain dicarboxylic acids. van Engen *et al*[38] reported that the *CYP4F2* polymorphism *(CYP4F2 p.433M)* increased the chances of acquiring CALD in male Caucasians (Table 1). They further demonstrated the functional impact of the *CYP4F2 p.433M* variant on cellular models, which showed reduced CYP4F2 protein level, led to a reduction in the metabolism of VLCFA through ω-oxidation.

**Inflammation-related genes:** Brain inflammation and the ensuing progressive inflammatory demyelination is characteristically found in the demyelinating forms of X-ALD. Acute inflammation occurs only in the CNS and not in other tissues of the affected cases[39]. Variants in the genes playing a role inflammation have been speculated to influence disease variability. The putative modifying genes could participate in an inflammatory response to the buildup of VLCFA or some other related metabolite in the brain. Since the pathology of the cerebral form is akin to that seen in multiple sclerosis (MS), some of the genetic factors involved in triggering inflammation in multiple sclerosis could also participate in the pathogenesis of X-ALD.

Genetic variants of specific major histocompatibility complex class II antigens ( *HLA-DRB1*) are reported to be associated with the risk for MS and could be suitable candidate modifiers for X-ALD. Berger *et al*[40] described a significant relationship between the *HLA-DRB1*16* allele and X-ALD. However, this allele did not show association with CALD, the inflammatory phenotype of X-ALD. The *DRB* genes are involved in the synthesis of peptides receptors playing a central role in the immune system.

Myelin oligodendrocyte glycoprotein (MOG) is the main target for demyelinating autoantibodies in MS. Schmidt *et al*[41] found increased serum anti-MOG in X-ALD cases and reported that these were linked with *MOG* (*TAAA*)*n* gene polymorphism but not with clinical types of ALD. Gomez- Lira and coworkers[42] identified six sequence variants in *MOG* gene: G15A, G511C, G520A, CTC repeats in exon1, 551168A→G and 551177C→T in X-ALD patients, but no frequency difference was observed in cases when compared to controls.

Tumor necrosis factor (TNF-α), a major pro-inflammatory cytokine, is involved in the pathogenesis of many neurological disorders including MS. TNF-α is capable of causing damage to the myelin sheath and oligodendrocytes and has also found to modulate the *MBP* (Myelin basic protein) gene promoter activity, *via* activation of NF-κB transcriptional factor in oligodendroglioma cells[43]. However, increased TNF-α bioactivity was not found to be associated with any allelic difference in the *TNF-a* gene (G- 308A).

Genetic variants of the cytokine, interleukin-6 (*IL-6*), such as the *IL-6 C*-allele which is a variable number tandem repeat polymorphism situated on the 3′ flanking region of the *IL-6* gene, is reported to be linked to late-onset Alzheimer's and MS, but no association was found with the different clinical phenotypes of X-ALD[41].

Neuroinflammation in CALD is suspected to be due to the involvement of different classes of lipids enriched in the VLCFA[2]; thus, the participation of CD1, a lipid antigen-presenting molecule, was speculated. Barbier *et al*[44] assessed the association between the genetic variants of CD1 molecules (*CD1A-E*) and the presence of neuroinflammation in X-ALD but found no association between them.

**Genes associated with methionine metabolism:** The pathological characteristic of the cerebral type of X-ALD is CNS demyelination. Demyelination starts in the mid of corpus callosum and advances outwards in both brain hemispheres. This leads to a gradual neurologic decline and death within 3 to 5 years[3].

The sulfur-containing amino acid, methionine, plays a vital metabolic role in providing methyl group required for DNA methylation, brain myelination, and precursors for the generation of glutathione taurine. S-adenosyl methionine (SAM), the active form of methionine, is a methyl donor. Deficiency of SAM can lead to demyelination in the CNS. Studies have reported variants of methionine metabolism as risk factors causing demyelination in X-ALD patients. Linnebank *et al*[45] studied the combined risk genotype, *i.e.* the occurrence of a minimum of one distinct genotype of three functional polymorphisms in genes associated with methionine metabolism, 5,10-methylenetetrahydrofolate reductase (*MTHFR*) c.677CT, methionine synthase ( *MTR)* c.2756AG, and transcobalamin 2 (*Tc2*) c.776CG, in 86 patients with various phenotypes of X-ALD. These authors reported that CCALD patients tended to have a higher prevalence of the combined risk genotype (46%) in comparison to the group with the benign variant "pure" AMN (33%; $P = 0.222$) due to a higher prevalence of the *MTR* (41% *vs* 22%, $P = 0.110$) and the *Tc2* risk genotype (18% *vs* 14%, $P = 0.675$). Moreover, this genotype was overrepresented in patients with AMN with CNS demyelination (AMN-cerebral) when compared to 49 AMN patients without CNS demyelination ("pure" AMN) and suggested that variations in genes associated with methionine metabolism might influence the phenotypic variability in X-ALD. Cystathionine β-synthase (*CBS*) is another important enzyme in the methionine metabolic pathway, and the *CBS* c.844_845ins68 variant may affect the availability or concentrations of activated methionine and glutathione. Linnebank and colleagues also found that *CBS* c.844_845ins68 insertion allele protected X-ALD patients from cerebral demyelination[46].

In another study of *CBS* c.844_855ins68, *MTR* c.2756A to G, and *TC2* c.776 C to G in 120 Chinese ALD patients, the frequency of only the GG genotype of the *TC2* c.776 C/G was more in those with brain demyelination than in controls[47]. TC2 is the transport carrier protein for cobalamin and methylcobalamin, the active form of cobalamin, a crucial cofactor necessary for the enzymatic activity of methionine synthase.

These results were further confirmed by Semmler *et al*[48] who genotyped eight polymorphisms in methionine metabolism genes, including *CBS* c.844_855ins68,

*MTHFR* c.677C>T, *MTR* c.2756A>G and *DHFR* c.594+59del19bp, and found *Tc2* c.776 GG genotype to be more prevalent in X-ALD cases with clinical features of brain demyelination compared to those without demyelination.

**Other potential genetic modifiers:** Reactive oxygen species (ROS) can trigger oxidative damage to DNA and proteins and ineffective oxidative phosphorylation, and this could result in dying-back axonopathy. Axonal degeneration in the spinal cord is typically observed in the AMN form of X-ALD. The mitochondrial superoxide dismutase *(SOD2*) is responsible for detoxifying ROS and is considered a modifying factor for the development of demyelination in X-ALD. A study reported that *SOD2* variant C47T and GTAC haplotype with reduced activity were associated with adolescent cerebral, adult cerebral X-ALD, and AMN- cerebral patients[49] (Table 1).

Bile acid metabolism occurs in the peroxisomes. An abnormal bile acid profile and mutations in the genes associated with the metabolism of bile acids such as *CYP7A1, CYP27A1, CYP7B1, HSD3B7, AKR1D1*, and *SLC27A52*, has been reported in a Polish AMN patient and these genes have been suggested as potential modifiers of X-ALD[50] (Table 1). However, more studies are required to confirm this association

Apolipoprotein E, a protein associating with lipid particles and functioning in lipoprotein-mediated lipid transport between organs, has three isoforms APOE2, APOE3 and APOE4 encoded by three alleles situated on a single gene locus. APOE3 protein maintains the blood-brain-barrier integrity (BBB) through the downregulation of cyclophilin A (CypA), a pro-inflammatory protein[51]. Male X-ALD patients bearing the *APOE4* genotype are reported to have greater cerebral involvement as determined by MRI severity score, lesser neurologic function, and elevated concentrations of matrix metalloproteinase-2 (MMP-2) in the cerebrospinal fluid compared to non-carriers[52]. The presence of the *APOE4* allele has been suggested to upregulate CypA leading to the activation of MMP-9 and loss of BBB integrity, leading to increased severity of cerebral disease in cerebral ALD[51].

### *Influence of epigenetic factors*

Epigenetic factors, too, can influence the onset of disease by inducing a subtle change in the gene expression without any notable alteration in the DNA sequence. Epigenetic alterations comprise DNA methylation, post-translational modifications of histones such as methylation, phosphorylation, acetylation, and post-transcriptional regulation by non-coding RNA.

DNA methylation acts as a regulatory mechanism for gene expression, and cell differentiation and various studies have demonstrated the association between change in DNA methylation and disease pathogenesis[53]. A study by Schlüter *et al*[54] compared the genome-wide DNA methylation pattern of unaffected frontal brain white matter of patients with CCALD and AMN with cerebral involvement and found hypermethylation of genes that are majorly involved in differentiation of oligodendrocytes including *MBP*, *CNP*, *MOG*, *PLP1* that can result to impaired differentiation of oligodendrocyte precursor cells to remyelinating oligodendrocyte and hypomethylation of genes associated with an immune function such as *IFITM1* and *CD59*. This supports the neuropathological evidence of lack of remyelination and immune activation noted in the cerebral form of X-ALD. This study also showed that combined methylation levels of *SPG20*, *UNC45A*, and *COL9A3* and combined expression levels of *ID4* and *MYRF* could be useful as biomarkers for differentiating CALD from AMN.

Aberrant expression of microRNAs (miRNAs), a group of small non-coding RNAs regulating post-transcriptional gene expression, has been suggested to play a significant part in the development of neuroinflammation and degeneration[55]. Shah *et al*[55] found decreased expression of miR-196a and increased expression of *ELOVL, IKKa*, *IKKβ*, *MAP4K3*, and *MAP3K2* in cerebral ALD compared to AMN and control fibroblasts, and suggested that the regulation of inflammatory signaling pathway in CALD brain occurs *via* miR-196a.

### *Other potential modifying factors*

Various host or cellular environmental factors may influence disease development in an individual. Oxidative stress is a common phenomenon reported in various neurodegenerative disorders, including X-ALD. Overproduction of free radicles results in lipid peroxidation, whose byproducts can cause deleterious damage to the cells[56]. Nury *et al*[57] observed reduced plasma levels of oxidative stress markers such as α-tocopherol, GSH, and docosahexaenoic acid (DHA) in different X-ALD phenotypes. These authors also showed that 7α-hydroxycholesterol, 7β-hydroxycholesterol, 7-ketocholesterol, and 9- and 13-hydroxyoctadecadienoic acids

were produced as a result of oxidative stress. Increased level of 7-ketocholesterol was found to cause overproduction of free radicles, activation of PRAP-1, and caspase 3 and elevated LC3-II/LC3-I and p62 in BV-12 microglia cells, indicating its ability to induce cell death[53]. A recent study demonstrated 7-ketocholesterol induced activation of PRAP-1 *via* NF-κB transforms microglial cells from a resting stage to an active stage, ultimately damaging the neurons. As 7-ketocholesterol induces oxidative stress, inflammation, and cell death, high levels could enhance peroxisomal dysfunction in microglial cells, promoting brain damage in the affected patients.

Jang *et al*[58] demonstrated the abnormal generation of cholesterol 25-hydroxylase and 25-hydroxycholesterol in CCALD patient-derived cell models and showed that 25-hydroxycholesterol aids the aggregation and activation of NLRP3 inflammasome, a caspase-1- activating multi-protein complex, resulting in increased formation of pro-inflammatory cytokines, IL-1β, IL-18[59]. 25-hydroxycholesterol has also been found to induce mitochondrial-dependent apoptosis of cells *via* the stimulation of glycogen synthesis kinase-3β (GSK-3β)/LXR pathway in the amyotrophic lateral sclerosis cell model[60]. This could also account for severe cerebral inflammatory demyelination, the hallmark of CCALD.

Trauma to the head has been speculated to trigger or worsen symptoms in X-ALD[61], and asymptomatic cases of X-ALD presenting with symptoms after head trauma have been reported. The inflammatory response following a traumatic brain injury, followed by mitochondrial dysfunction, oxidative stress, and disruption of the BBB, has been suggested to activate cerebral inflammatory demyelination resulting in the appearance of symptoms[62].

The major difference between the different X-ALD phenotypes is the presence or absence of neuroinflammation and cerebral demyelination. The inflammatory response in the brain is believed to begin after the abnormal accumulation of VLCFA. ALDP deficiency has been shown to induce alteration in brain endothelial cells favoring the migration of leukocytes by downregulating the expression of c-Myc, leading to a reduction in the expression of cell surface tight junction proteins CLDN5 and ZO1 and increased expression of cell adhesion molecule ICAM-1 and MMP9[63]. We speculate that the migration of immune cells into the brain could be a rate-limiting step in the induction of cerebral demyelination. The onset of the migration across the BBB could be precipitated by various environmental triggers, genetic or epigenetic factors and ABCD1 deficiency acting either alone or in concert, marking the onset of brain inflammation leading to cerebral symptoms (Figure 2). Further, the absence of neuroinflammation in "pure AMN" and "Addison's only" phenotype could also possibly be due to the involvement of modifiers that are protective against the VLCFA toxicity in the brain. For instance, it is commonly known that MMP2 and MMP9 are required for the migration of the immune cells across the endothelial basal membrane and parenchymal border, respectively. However, the delay in the synthesis and secretion of MMP2 and MMP9 could delay the migration process and, ultimately, the disease onset. Genetic factors such as *APOE4*, which is shown to be associated with cerebral involvement in young males, are also associated with increased expression of MMP9 *via* cyclophilin A, leading to BBB leakiness. Thus, a complex interplay between multiple determinants could affect the onset and severity of the disease symptoms.

## CONCLUSION

The role of different modifiers influencing disease phenotypes has been described in various metabolic disorders. Therefore, though X-ALD is a monogenic disorder, other genetic factors, along with the environmental triggers, may be responsible for the severity and penetrance of the disease. Although numerous studies have made efforts to understand different genetic, epigenetic, and environmental factors in X-ALD, the exact cause of phenotypic differences in X-ALD patients with the same genotype is not clear. Improved knowledge of these factors will allow identification of patients prone to developing a particular form or clinical type of X-ALD. Besides, detailed elucidation of the association of different potential modifiers with the clinical heterozygosity in X-ALD is crucial for understanding the disease pathogenesis and for developing novel therapeutic strategies. With the introduction of neonatal screening for X-ALD, X-ALD modifiers will become increasingly essential to categorize patients who are likely to develop cerebral demyelination and plan appropriate management of these patients.

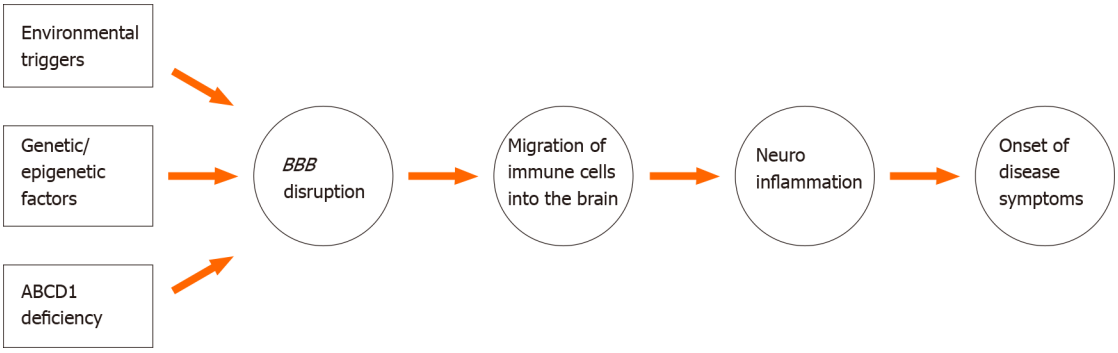


**Figure 2 Factors determining the onset of symptoms of cerebral demyelination.** Migration of immune cells into the brain could be a rate-limiting step in the appearance of symptoms of cerebral demyelination. Onset of the migration across the blood brain barrier could be precipitated by environmental triggers, genetic or epigenetic factors and *ATP Binding Cassette Subfamily D Member 1* (*ABCD1*) deficiency acting either alone or in concert, marking the onset of brain inflammation leading to cerebral symptoms.

## REFERENCES


1 **Moser HW**, Mahmood A, Raymond GV. X-linked adrenoleukodystrophy. *Nat Clin Pract Neurol* 2007; **3**: 140-151 [PMID: 17342190 DOI: 10.1038/ncpneuro0421]

2 **Kemp S**, Wanders R. Biochemical aspects of X-linked adrenoleukodystrophy. *Brain Pathol* 2010; **20**: 831-837 [PMID: 20626744 DOI: 10.1111/j.1750-3639.2010.00391.x]

3 **Berger J**, Forss-Petter S, Eichler FS. Pathophysiology of X-linked adrenoleukodystrophy. *Biochimie* 2014; **98**: 135-142 [PMID: 24316281 DOI: 10.1016/j.biochi.2013.11.023]

4 **Kemp S**, Pujol A, Waterham HR, van Geel BM, Boehm CD, Raymond GV, Cutting GR, Wanders RJ, Moser HW. ABCD1 mutations and the X-linked adrenoleukodystrophy mutation database: role in diagnosis and clinical correlations. *Hum Mutat* 2001; **18**: 499-515 [PMID: 11748843 DOI: 10.1002/humu.1227]

5 **Parisi LR**, Sowlati-Hashjin S, Berhane IA, Galster SL, Carter KA, Lovell JF, Chemler SR, Karttunen M, Atilla-Gokcumen GE. Membrane Disruption by Very Long Chain Fatty Acids during Necroptosis. *ACS Chem Biol* 2019; **14**: 2286-2294 [PMID: 31490656 DOI: 10.1021/acschembio.9b00616]

6 **Khan M**, Singh J, Gilg AG, Uto T, Singh I. Very long-chain fatty acid accumulation causes lipotoxic response *via* 5-lipoxygenase in cerebral adrenoleukodystrophy. *J Lipid Res* 2010; **51**: 1685-1695 [PMID: 20173212 DOI: 10.1194/jlr.M002329]

7 **van de Beek MC**, Ofman R, Dijkstra I, Wijburg F, Engelen M, Wanders R, Kemp S. Lipid-induced endoplasmic reticulum stress in X-linked adrenoleukodystrophy. *Biochim Biophys Acta Mol Basis Dis* 2017; **1863**: 2255-2265 [PMID: 28666219 DOI: 10.1016/j.bbadis.2017.06.003]

8 **Launay N**, Ruiz M, Fourcade S, Schlüter A, Guilera C, Ferrer I, Knecht E, Pujol A. Oxidative stress regulates the ubiquitin-proteasome system and immunoproteasome functioning in a mouse model of X-adrenoleukodystrophy. *Brain* 2013; **136**: 891-904 [PMID: 23436506 DOI: 10.1093/brain/aws370]

9 **Powers JM**, DeCiero DP, Ito M, Moser AB, Moser HW. Adrenomyeloneuropathy: a neuropathologic review featuring its noninflammatory myelopathy. *J Neuropathol Exp Neurol* 2000; **59**: 89-102 [PMID: 10749098 DOI: 10.1093/jnen/59.2.89]

10 **van Geel BM**, Assies J, Wanders RJ, Barth PG. X linked adrenoleukodystrophy: clinical presentation, diagnosis, and therapy. *J Neurol Neurosurg Psychiatry* 1997; **63**: 4-14 [PMID: 9221959 DOI: 10.1136/jnnp.63.1.4]

11 **Jangouk P**, Zackowski KM, Naidu S, Raymond GV. Adrenoleukodystrophy in female heterozygotes: underrecognized and undertreated. *Mol Genet Metab* 2012; **105**: 180-185 [PMID: 22112817 DOI: 10.1016/j.ymgme.2011.11.001]

12 **Hershkovitz E**, Narkis G, Shorer Z, Moser AB, Watkins PA, Moser HW, Manor E. Cerebral X-linked adrenoleukodystrophy in a girl with Xq27-Ter deletion. *Ann Neurol* 2002; **52**: 234-237 [PMID: 12210797 DOI: 10.1002/ana.10248]

13 **Chen X**, Chen Z, Huang D, Liu X, Gui Q, Yu S. Adult cerebral adrenoleukodystrophy and Addison's disease in a female carrier. *Gene* 2014; **544**: 248-251 [PMID: 24768737 DOI: 10.1016/j.gene.2014.04.056]

14 **Maier EM**, Kammerer S, Muntau AC, Wichers M, Braun A, Roscher AA. Symptoms in carriers of adrenoleukodystrophy relate to skewed X inactivation. *Ann Neurol* 2002; **52**: 683-688 [PMID: 12402273 DOI: 10.1002/ana.10376]

15 **Wang Z**, Yan A, Lin Y, Xie H, Zhou C, Lan F. Familial skewed x chromosome inactivation in adrenoleukodystrophy manifesting heterozygotes from a Chinese pedigree. *PLoS One* 2013; **8**: e57977 [PMID: 23469258 DOI: 10.1371/journal.pone.0057977]

16 **Moser HW**, Moser AB, Smith KD, Bergin A, Borel J, Shankroff J, Stine OC, Merette C, Ott J, Krivit W. Adrenoleukodystrophy: phenotypic variability and implications for therapy. *J Inherit Metab Dis* 1992; **15**: 645-664 [PMID: 1528023 DOI: 10.1007/BF01799621]

17 **Simpson RH**, Rodda J, Reinecke CJ. Adrenoleukodystrophy in a mother and son. *J Neurol Neurosurg Psychiatry* 1987; **50**: 1165-1172 [PMID: 2822858 DOI: 10.1136/jnnp.50.9.1165]

18 **Elrington GM**, Bateman DE, Jeffrey MJ, Lawton NF. Adrenoleukodystrophy: heterogeneity in two brothers. *J Neurol Neurosurg Psychiatry* 1989; **52**: 310-313 [PMID: 2538572 DOI: 10.1136/jnnp.52.3.310]

19 **Korenke GC**, Fuchs S, Krasemann E, Doerr HG, Wilichowski E, Hunneman DH, Hanefeld F. Cerebral adrenoleukodystrophy (ALD) in only one of monozygotic twins with an identical ALD genotype. *Ann Neurol*

1996; **40**: 254-257 [PMID: 8773611 DOI: 10.1002/ana.410400221]
20 **Gosalakkal J**, Balky AP. Intra familial phenotypical variations in adrenoleukodystrophy. *Neurol India* 2010; **58**: 109-111 [PMID: 20228476 DOI: 10.4103/0028-3886.60418]
21 **Soardi FC**, Esquiaveto-Aun AM, Guerra-Júnior G, Lemos-Marini SH, Mello MP. Phenotypic variability in a family with x-linked adrenoleukodystrophy caused by the p.Trp132Ter mutation. *Arq Bras Endocrinol Metabol* 2010; **54**: 738-743 [PMID: 21340162 DOI: 10.1590/S0004-27302010000800013]
22 **Kallabi F**, Ellouz E, Tabebi M, Ben Salah G, Kaabechi N, Keskes L, Triki C, Kamoun H. Phenotypic variability in a Tunisian family with X-linked adrenoleukodystrophy caused by the p.Gln316Pro novel mutation. *Clin Chim Acta* 2016; **453**: 141-146 [PMID: 26686776 DOI: 10.1016/j.cca.2015.12.014]
23 **van Geel BM**, Assies J, Weverling GJ, Barth PG. Predominance of the adrenomyeloneuropathy phenotype of X-linked adrenoleukodystrophy in The Netherlands: a survey of 30 kindreds. *Neurology* 1994; **44**: 2343-2346 [PMID: 7991123 DOI: 10.1212/WNL.44.12.2343]
24 **Sobue G**, Ueno-Natsukari I, Okamoto H, Connell TA, Aizawa I, Mizoguchi K, Honma M, Ishikawa G, Mitsuma T, Natsukari N. Phenotypic heterogeneity of an adult form of adrenoleukodystrophy in monozygotic twins. *Ann Neurol* 1994; **36**: 912-915 [PMID: 7998779 DOI: 10.1002/ana.410360617]
25 **Kemp S**, Ligtenberg MJ, van Geel BM, Barth PG, Wolterman RA, Schoute F, Sarde CO, Mandel JL, van Oost BA, Bolhuis PA. Identification of a two base pair deletion in five unrelated families with adrenoleukodystrophy: a possible hot spot for mutations. *Biochem Biophys Res Commun* 1994; **202**: 647-653 [PMID: 8048932 DOI: 10.1006/bbrc.1994.1979]
26 **Kok F**, Neumann S, Sarde CO, Zheng S, Wu KH, Wei HM, Bergin J, Watkins PA, Gould S, Sack G. Mutational analysis of patients with X-linked adrenoleukodystrophy. *Hum Mutat* 1995; **6**: 104-115 [PMID: 7581394 DOI: 10.1002/humu.1380060203]
27 **Mosser J**, Douar AM, Sarde CO, Kioschis P, Feil R, Moser H, Poustka AM, Mandel JL, Aubourg P. Putative X-linked adrenoleukodystrophy gene shares unexpected homology with ABC transporters. *Nature* 1993; **361**: 726-730 [PMID: 8441467 DOI: 10.1038/361726a0]
28 **Gallati S**. Disease-modifying genes and monogenic disorders: experience in cystic fibrosis. *Appl Clin Genet* 2014; **7**: 133-146 [PMID: 25053892 DOI: 10.2147/TACG.S18675]
29 **Nadeau JH**. Modifier genes in mice and humans. *Nat Rev Genet* 2001; **2**: 165-174 [PMID: 11256068 DOI: 10.1038/35056009]
30 **Smith KD**, Sack G, Beaty T, Bergin A, Naidu S, Moser A MH. A genetic-basis for the multiple phenotypes of X-linked adrenoleukodystrophy. *Am J Hum Genet* 1991; **49**: 165-165
31 **Sassa T**, Kihara A. Metabolism of very long-chain Fatty acids: genes and pathophysiology. *Biomol Ther (Seoul)* 2014; **22**: 83-92 [PMID: 24753812 DOI: 10.4062/biomolther.2014.017]
32 **Smith KD**, Kemp S, Braiterman LT, Lu JF, Wei HM, Geraghty M, Stetten G, Bergin JS, Pevsner J, Watkins PA. X-linked adrenoleukodystrophy: genes, mutations, and phenotypes. *Neurochem Res* 1999; **24**: 521-535 [PMID: 10227685 DOI: 10.1023/A:1022535930009]
33 **Asheuer M**, Bieche I, Laurendeau I, Moser A, Hainque B, Vidaud M, Aubourg P. Decreased expression of ABCD4 and BG1 genes early in the pathogenesis of X-linked adrenoleukodystrophy. *Hum Mol Genet* 2005; **14**: 1293-1303 [PMID: 15800013 DOI: 10.1093/hmg/ddi140]
34 **Maier EM**, Mayerhofer PU, Asheuer M, Köhler W, Rothe M, Muntau AC, Roscher AA, Holzinger A, Aubourg P, Berger J. X-linked adrenoleukodystrophy phenotype is independent of ABCD2 genotype. *Biochem Biophys Res Commun* 2008; **377**: 176-180 [PMID: 18834860 DOI: 10.1016/j.bbrc.2008.09.092]
35 **Matsukawa T**, Asheuer M, Takahashi Y, Goto J, Suzuki Y, Shimozawa N, Takano H, Onodera O, Nishizawa M, Aubourg P, Tsuji S. Identification of novel SNPs of ABCD1, ABCD2, ABCD3, and ABCD4 genes in patients with X-linked adrenoleukodystrophy (ALD) based on comprehensive resequencing and association studies with ALD phenotypes. *Neurogenetics* 2011; **12**: 41-50 [PMID: 20661612 DOI: 10.1007/s10048-010-0253-6]
36 **Kemp S**, Valianpour F, Denis S, Ofman R, Sanders RJ, Mooyer P, Barth PG, Wanders RJ. Elongation of very long-chain fatty acids is enhanced in X-linked adrenoleukodystrophy. *Mol Genet Metab* 2005; **84**: 144-151 [PMID: 15670720 DOI: 10.1016/j.ymgme.2004.09.015]
37 **Ofman R**, Dijkstra IM, van Roermund CW, Burger N, Turkenburg M, van Cruchten A, van Engen CE, Wanders RJ, Kemp S. The role of ELOVL1 in very long-chain fatty acid homeostasis and X-linked adrenoleukodystrophy. *EMBO Mol Med* 2010; **2**: 90-97 [PMID: 20166112 DOI: 10.1002/emmm.201000061]
38 **van Engen CE**, Ofman R, Dijkstra IM, van Goethem TJ, Verheij E, Varin J, Vidaud M, Wanders RJ, Aubourg P, Kemp S, Barbier M. CYP4F2 affects phenotypic outcome in adrenoleukodystrophy by modulating the clearance of very long-chain fatty acids. *Biochim Biophys Acta* 2016; **1862**: 1861-1870 [PMID: 27425035 DOI: 10.1016/j.bbadis.2016.07.006]
39 **Moser HW**, Smith KD, Watkins PA, Powers J MA. X-linked adrenoleukodystrophy. In: Scriver CR, Beaudet AL, Sly WS, Valle D, editors. The metabolic and molecular bases of inherited disease (Vol. 4). New York: McGraw Hill, 2001: 3257-3301
40 **Berger J**, Bernheimer H, Faé I, Braun A, Roscher A, Molzer B, Fischer G. Association of X-linked adrenoleukodystrophy with HLA DRB1 alleles. *Biochem Biophys Res Commun* 1995; **216**: 447-451 [PMID: 7488132 DOI: 10.1006/bbrc.1995.2643]
41 **Schmidt S**, Marrosu GM, Kölsch H, Haase CG, Ferenczik S, Sokolowski P, Köhler W, Schmidt M, Papassotiropoulos A, Heun R, Grosse-Wilde H, Klockgether T. Genetic variations and humoral immune responses to myelin oligodendroglia glycoprotein in adult phenotypes of X-linked adrenoleukodystrophy. *J Neuroimmunol* 2003; **135**: 148-153 [PMID: 12576235 DOI: 10.1016/S0165-5728(02)00445-9]
42 **Gomez-Lira M**, Marzari MG, Uziel G, Pignatti P, Rizzuto N, Salviati A. Myelin oligodendrocyte glycoprotein (MOG) polymorphisms and adrenoleukodystrophy. *J Neuroimmunol* 2000; **111**: 245-247 [PMID: 11063846 DOI: 10.1016/S0165-5728(00)00367-2]
43 **Huang CJ**, Nazarian R, Lee J, Zhao PM, Espinosa-Jeffrey A, de Vellis J. Tumor necrosis factor modulates transcription of myelin basic protein gene through nuclear factor kappa B in a human oligodendroglioma cell line. *Int J Dev Neurosci* 2002; **20**: 289-296 [PMID: 12175864 DOI: 10.1016/S0736-5748(02)00022-9]

44 **Barbier M**, Sabbagh A, Kasper E, Asheuer M, Ahouansou O, Pribill I, Forss-Petter S, Vidaud M, Berger J, Aubourg P. CD1 gene polymorphisms and phenotypic variability in X-linked adrenoleukodystrophy. *PLoS One* 2012; **7**: e29872 [PMID: 22253809 DOI: 10.1371/journal.pone.0029872]

45 **Linnebank M**, Kemp S, Wanders RJ, Kleijer WJ, van der Sterre ML, Gärtner J, Fliessbach K, Semmler A, Sokolowski P, Köhler W, Schlegel U, Schmidt S, Klockgether T, Wüllner U. Methionine metabolism and phenotypic variability in X-linked adrenoleukodystrophy. *Neurology* 2006; **66**: 442-443 [PMID: 16476952 DOI: 10.1212/01.wnl.0000196491.42058.6f]

46 **Linnebank M**, Semmler A, Kleijer WJ, van der Sterre ML, Gärtner J, Fliessbach K, Sokolowski P, Köhler W, Schlegel U, Klockgether T, Wanders RJ, Schmidt S, Wüllner U, Kemp S. The cystathionine beta-synthase variant c.844_845ins68 protects against CNS demyelination in X-linked adrenoleukodystrophy. *Hum Mutat* 2006; **27**: 1063-1064 [PMID: 16941496 DOI: 10.1002/humu.9459]

47 **Cao GN**, Bao XH, Xiong H, Wu Y, Wu XR. [Association of genetic polymorphisms in methionine metabolism genes with X-linked adrenoleukodystrophy]. *Zhonghua Yi Xue Yi Chuan Xue Za Zhi* 2011; **28**: 279-282 [PMID: 21644223 DOI: 10.3760/cma.j.issn.1003-9406.2011.03.009]

48 **Semmler A**, Bao X, Cao G, Köhler W, Weller M, Aubourg P, Linnebank M. Genetic variants of methionine metabolism and X-ALD phenotype generation: results of a new study sample. *J Neurol* 2009; **256**: 1277-1280 [PMID: 19353223 DOI: 10.1007/s00415-009-5114-6]

49 **Brose RD**, Avramopoulos D, Smith KD. SOD2 as a potential modifier of X-linked adrenoleukodystrophy clinical phenotypes. *J Neurol* 2012; **259**: 1440-1447 [PMID: 22218650 DOI: 10.1007/s00415-011-6371-8]

50 **Platek T**, Orso E, Zapała B, Polus A, Kieć-Wilk B, Piwowar M, Chojnacka M, Ciałowicz U, Malczewska-Malec M, Schmitz G, Solnica B, Dembińska-Kieć A. Case report of dysregulation of primary bile acid synthesis in a family with X-linked adrenoleukodystrophy. *Medicine (Baltimore)* 2018; **97**: e13353 [PMID: 30544401 DOI: 10.1097/MD.0000000000013353]

51 **Bell RD**, Winkler EA, Singh I, Sagare AP, Deane R, Wu Z, Holtzman DM, Betsholtz C, Armulik A, Sallstrom J, Berk BC, Zlokovic BV. Apolipoprotein E controls cerebrovascular integrity *via* cyclophilin A. *Nature* 2012; **485**: 512-516 [PMID: 22622580 DOI: 10.1038/nature11087]

52 **Orchard PJ**, Markowski TW, Higgins L, Raymond GV, Nascene DR, Miller WP, Pierpont EI, Lund TC. Association between APOE4 and biomarkers in cerebral adrenoleukodystrophy. *Sci Rep* 2019; **9**: 7858 [PMID: 31133696 DOI: 10.1038/s41598-019-44140-3]

53 **Fedotova EY**, Illarioshkin SN. DNA Methylation in Neurodegenerative Diseases. *Russ J Gene***55**: 271–277 [DOI: 10.1134/S1022795419030062]

54 **Schlüter A**, Sandoval J, Fourcade S, Díaz-Lagares A, Ruiz M, Casaccia P, Esteller M, Pujol A. Epigenomic signature of adrenoleukodystrophy predicts compromised oligodendrocyte differentiation. *Brain Pathol* 2018; **28**: 902-919 [PMID: 29476661 DOI: 10.1111/bpa.12595]

55 **Shah N**, Singh I. MicroRNA Profiling Identifies miR-196a as Differentially Expressed in Childhood Adrenoleukodystrophy and Adult Adrenomyeloneuropathy. *Mol Neurobiol* 2017; **54**: 1392-1403 [PMID: 26843114 DOI: 10.1007/s12035-016-9746-0]

56 **Ranea-Robles P**, Launay N, Ruiz M, Calingasan NY, Dumont M, Naudí A, Portero-Otín M, Pamplona R, Ferrer I, Beal MF, Fourcade S, Pujol A. Aberrant regulation of the GSK-3β/NRF2 axis unveils a novel therapy for adrenoleukodystrophy. *EMBO Mol Med* 2018; **10**: e8604 [PMID: 29997171 DOI: 10.15252/emmm.201708604]

57 **Nury T**, Zarrouk A, Ragot K, Debbabi M, Riedinger JM, Vejux A, Aubourg P, Lizard G. 7-Ketocholesterol is increased in the plasma of X-ALD patients and induces peroxisomal modifications in microglial cells: Potential roles of 7-ketocholesterol in the pathophysiology of X-ALD. *J Steroid Biochem Mol Biol* 2017; **169**: 123-136 [PMID: 27041118 DOI: 10.1016/j.jsbmb.2016.03.037]

58 **Jang J**, Park S, Jin Hur H, Cho HJ, Hwang I, Pyo Kang Y, Im I, Lee H, Lee E, Yang W, Kang HC, Won Kwon S, Yu JW, Kim DW. 25-hydroxycholesterol contributes to cerebral inflammation of X-linked adrenoleukodystrophy through activation of the NLRP3 inflammasome. *Nat Commun* 2016; **7**: 13129 [PMID: 27779191 DOI: 10.1038/ncomms13129]

59 **Yang Y**, Wang H, Kouadir M, Song H, Shi F. Recent advances in the mechanisms of NLRP3 inflammasome activation and its inhibitors. *Cell Death Dis* 2019; **10**: 128 [PMID: 30755589 DOI: 10.1038/s41419-019-1413-8]

60 **Vejux A**, Lizard G. Cytotoxic effects of oxysterols associated with human diseases: Induction of cell death (apoptosis and/or oncosis), oxidative and inflammatory activities, and phospholipidosis. *Mol Aspects Med* 2009; **30**: 153-170 [PMID: 19248805 DOI: 10.1016/j.mam.2009.02.006]

61 **Budhram A**, Pandey SK. Activation of Cerebral X-linked Adrenoleukodystrophy After Head Trauma. *Can J Neurol Sci* 2017; **44**: 597-598 [PMID: 28534457 DOI: 10.1017/cjn.2017.52]

62 **Bouquet F**, Dehais C, Sanson M, Lubetzki C, Louapre C. Dramatic worsening of adult-onset X-linked adrenoleukodystrophy after head trauma. *Neurology* 2015; **85**: 1991-1993 [PMID: 26537054 DOI: 10.1212/WNL.0000000000002173]

63 **Musolino PL**, Gong Y, Snyder JM, Jimenez S, Lok J, Lo EH, Moser AB, Grabowski EF, Frosch MP, Eichler FS. Brain endothelial dysfunction in cerebral adrenoleukodystrophy. *Brain* 2015; **138**: 3206-3220 [PMID: 26377633 DOI: 10.1093/brain/awv250]

64 **McGuinness MC**, Griffin DE, Raymond GV, Washington CA, Moser HW, Smith KD. Tumor necrosis factor-alpha and X-linked adrenoleukodystrophy. *J Neuroimmunol* 1995; **61**: 161-169 [PMID: 7593551 DOI: 10.1016/0165-5728(95)00084-F]

65 **McGuinness MC**, Powers JM, Bias WB, Schmeckpeper BJ, Segal AH, Gowda VC, Wesselingh SL, Berger J, Griffin DE, Smith KD. Human leukocyte antigens and cytokine expression in cerebral inflammatory demyelinative lesions of X-linked adrenoleukodystrophy and multiple sclerosis. *J Neuroimmunol* 1997; **75**: 174-182 [PMID: 9143252 DOI: 10.1016/S0165-5728(97)00020-9]

World Journal of Biological Chemistry

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

*World J Biol Chem* 2020 November 27; 11(3): 112-118

DOI: 10.4331/wjbc.v11.i3.112

ISSN 1949-8454 (online)

ORIGINAL ARTICLE

Observational Study

# Prevalence, serotyping and drug susceptibility patterns of *Escherichia coli* isolates from kidney transplanted patients with urinary tract infections

Atefeh Najafi Khah, Mojdeh Hakemi-Vala, Shiva Samavat, Mohammad Javad Nasiri

**ORCID number:** Atefeh Najafi Khah 0000-0002-4677-4920; Mojdeh Hakemi-Vala 0000-0002-8355-6885; Shiva Samavat 0000-0001-6707-7844; Mohammad Javad Nasiri 0000-0002-3279-0671.

**Author contributions:** Hakemi-Vala M proposed the subject of the project, supervised the proposal, practical steps, revised the draft, and submitted the article to this journal; Samavat S, as the urologist in Labafi Nejad Hospital, introduced the patients who were compatible with this subject; Najafi Khah A is an MSc student in medical microbiology and this paper is part of her thesis, she also carried out all practical processes such as sampling, cooperated with two private clinical laboratories and drafted the paper; Nasiri MJ, a consultant, contributed to statistical analysis, paper preparation, and revision, data collection and analysis.

**Supported by** Research Department of School of Medicine Shahid Beheshti University of Medical Sciences, No. 17920, and accepted by the ethic committee, Code. IR.SBMU. MSP.REC.1398.349.

**Atefeh Najafi Khah, Mojdeh Hakemi-Vala, Mohammad Javad Nasiri,** Department of Microbiology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran 1985717443, Iran

**Shiva Samavat,** Department of Adult Nephrology, School of Medicine, Shahid Labbafinezhad Hospital, Shahid Beheshti University of Medical Sciences, Tehran 1666694516, Iran

**Corresponding author:** Mojdeh Hakemi-Vala, PhD, Associate Professor, Department of Microbiology, School of Medicine, Shahid Beheshti University of Medical Sciences, Kodakyar St, Daneshjo Blvd, Velenjak Tehran, Tehran 1985717443, Iran. m.hakemi@sbmu.ac.ir

## Abstract

### *BACKGROUND*

Extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* (*E. coli*) are among the main pathogens in urinary tract infections (UTIs) among kidney transplant patients (KTPs).

### *AIM*

To estimate the prevalence of ESBL-producing *E. coli* in KTPs and to evaluate the most prevalent serotypes and antibacterial susceptibility patterns of isolated bacteria in Tehran, Iran.

### *METHODS*

A total of 60 clinical isolates of uropathogenic *E. coli* were collected from 3 kidney transplant centers from April to May 2019. Antimicrobial susceptibility testing was performed by the disk diffusion method as recommended by the Clinical Laboratory and Standards Institute. The serotyping of *E. coli* isolates was performed by the slide agglutination method. The presence of $bla_{TEM}$, $bla_{SHV}$, and $bla_{CTX-M}$ genes was evaluated by polymerase chain reaction.

### *RESULTS*

The frequency of ESBL-producing *E. coli* in KTPs was found to be 33.4%. All of the 60 *E. coli* isolates were found to be susceptible to doripenem (100%) and ertapenem (100%). High resistance rates to ampicillin (86%), cefotaxime (80%), and cefazolin (77%) were also documented. The most frequent serotypes were serotype I (50%), serotype II (15%), serotype III (25%), and serotype VI (10%). The gene most frequently found was $bla_{TEM}$ (55%), followed by $bla_{CTX-M}$ (51%) and $bla_{SHV}$

**Institutional review board statement:** This paper is extracted from a project which was accepted by the research committee.

**Informed consent statement:** All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

**Conflict-of-interest statement:** This project has received a grant from the Research Department of School of Medicine Shahid Beheshti University of Medical Sciences, No. 17920, Tehran, Iran.

**Data sharing statement:** No additional data are available.

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



**Manuscript source:** Invited manuscript

**Specialty type:** Biochemistry and molecular biology

**Country/Territory of origin:** Iran

**Peer-review report's scientific quality classification**

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

**Received:** April 26, 2020

(41%).

*CONCLUSION*

Molecular analysis showed that $bla_{TEM}$ was the most common ESBL-encoding gene. The high resistance to β-lactams antibiotics (*i.e.*, ampicillin, cefotaxime, and cefazolin) found in *E. coli* from KTPs with UTIs remains a serious clinical challenge. Further efforts to control ESBL-producing *E. coli* should include the careful use of all antibiotics as well as barrier precautions to reduce spread.

**Key Words:** Kidney transplantation; Urinary tract infection; Drug resistance; *Escherichia coli*; Serotyping; β-Lactamase



**Core Tip:** Extended-spectrum β-lactamases (ESBLs)-producing *Escherichia coli* (*E. coli*) are among the main pathogens in urinary tract infections among kidney transplant patients (KTPs). The aims of this study were: To estimate the prevalence of ESBL-producing *E. coli* in KTPs, and to evaluate the most prevalent serotypes and antibacterial susceptibility patterns of isolated bacteria in Tehran, Iran. The most important findings were: (1) The frequency of ESBL-producing *E. coli* in KTPs was 33.4%; (2) High resistance rates to ampicillin (86%) and cefotaxime (80%) were documented; (3) The most frequent serotype was serotype I (50%); (4) The most frequently found related gene was *blaTEM* (55%); and (5) Further efforts to control ESBL-producing *E. coli* should include the careful use of all antibiotics as well as barrier precautions to reduce spread.

**Citation:** Najafi Khah A, Hakemi-Vala M, Samavat S, Nasiri MJ. Prevalence, serotyping and drug susceptibility patterns of *Escherichia coli* isolates from kidney transplanted patients with urinary tract infections. *World J Biol Chem* 2020; 11(3): 112-118
**URL:** https://www.wjgnet.com/1949-8454/full/v11/i3/112.htm
**DOI:** https://dx.doi.org/10.4331/wjbc.v11.i3.112

## INTRODUCTION

Urinary tract infection (UTI) remains one of the most common bacterial infections in kidney transplant patients (KTPs)[1,2]. *Escherichia coli* (*E. coli*) is one of the main uropathogens isolated from KTPs with UTIs[3]. Recently, several studies have reported a high incidence of extended-spectrum β-lactamases (ESBLs)-producing *E. coli* among KTPs[4]. Infections caused by ESBL-producing bacteria are usually associated with increased morbidity and mortality[5-7]. Therefore, UTI caused by ESBL-producing *E. coli* in KTPs is an important challenge in healthcare settings.

The ESBL-producing strains are resistant to all penicillins, cephalosporins (including first-, second-, and third-generation) and aztreonam. This event occurs due to the production of CTX-M, TEM, and SHV β-lactamases which are encoded by $bla_{CTX-M}$, $bla_{SHV}$, and $bla_{TEM}$ genes, respectively[5-7]. To date, several studies have reported the rates of ESBL-producing *E. coli* in Iran; however, very few studies have evaluated ESBL-producing bacteria in KTPs or their antimicrobial susceptibility profiles. Therefore, the aims of this study were to estimate the prevalence of ESBL-producing *E. coli* in KTPs, to serotype the ESBL-producing *E. coli*, and to identify the antibacterial susceptibility patterns of isolated bacteria in Tehran, Iran.

## MATERIALS AND METHODS

### *Setting and samples*

In this study, urine samples were collected using the mid-stream clean catch method. A total of 60 *E. coli* isolates from 60 KTPs referred to Labofinejad Hospital and two private laboratories, Yekta and Gholhak, were collected from April to May 2019. All

**Peer-review started:** April 26, 2020
**First decision:** June 7, 2020
**Revised:** August 24, 2020
**Accepted:** September 25, 2020
**Article in press:** September 25, 2020
**Published online:** November 27, 2020

**P-Reviewer:** Exbrayat JM
**S-Editor:** Huang P
**L-Editor:** Webster JR
**P-Editor:** Ma YJ

isolates were confirmed as *E. coli* by standard bacteriologic methods and kept in 10% glycerol and TSB at -70°C for further evaluation.

### Detection of ESBLs

ESBL production was detected according to the Clinical Laboratory and Standards Institute (CLSI) confirmatory test using cefotaxime 30 mg and ceftazidime (CAZ) 30 mg disks alone and in combination with clavulanic acid (CA) 10 mg[8]. The test was considered positive when an increase in the growth-inhibitory zone around either the cefotaxime or the CAZ disk with CA was 5 mm or greater than the diameter around cefotaxime or CAZ alone[9]. *E. coli* ATCC 25922 and *Klebsiella. pneumonia*e ATCC 700603 were used as negative and positive controls, respectively.

### Antimicrobial susceptibility testing

Antimicrobial susceptibility testing (AST) was performed by the disk diffusion method on Mueller-Hinton agar as recommended by the CLSI[10]. The tested antibiotics were purchased from Mast (England) or Rosco (Denmark) companies and were used for AST: Ceftriaxone 30 mg, cefotaxime 30 mg, cefixime 30 mg, cefazolin 30 µg, cephalexin 30 mg from Rosco Company and ampicillin 10 µg, ampicillin-sulbactam 20/10 µg, piperacillin/tazobactam 100/10 µg, cefpodoxime 30 µg, doripenem 10 µg, imipenem 10 µg, ertapenem 10 µg, meropenem 10 µg, gentamicin 10 µg, tobramycin 10 µg, amikacin 30 µg, ciprofloxacin 5 µg, trimethoprim 5 µg, and nitrofurantoine 200 µg from Mast Company, respectively.

A bacterial suspension with turbidity equal to a homemade 0.5 MacFarland standard ($1.5 \times 10^8$ CFU/mL) was prepared for each bacterial isolate, a bacterial lawn was performed on a Mueller Hinton agar plate using a sterile cotton swab and selected antibiotic disks were placed on the agar plate with sterile forceps. The plates were then incubated at 37°C for 24 h. The diameter of the zone of inhibition was measured and the results were reported as susceptible (S), resistant (R) or intermediate (I) based on the CLSI criteria[11]. *Escherichia coli* ATCC 25922 was used as a control.

### Serotyping

Agglutination (Bahar Afshan_Iran) reactions were performed in triplicate following the manufacturer's protocol: 25 µL of test solution and 25 µL of bacterial suspension were added to a black slide. They were then thoroughly mixed, and the slide was incubated for 5 min at room temperature on a rotator set to 100 rpm[12].

### DNA extraction and polymerase chain reaction method

A 1000 µL aliquot of cell suspension containing $10^7$ cells/mL was transferred to microtubes and incubated at 100°C in a boiling water-bath for 5 min. The suspension containing DNA was vigorously homogenized by vortex for 10 s and the tube was frozen on ice. The DNA sample was stored at -18°C[13].

*β-Lactamase* genes were amplified by the polymerase chain reaction (PCR) using a panel of primers for the detection of $bla_{TEM}$, $bla_{SHV,}$ and $bla_{CTX-M}$ genes[13]. PCR amplification of $bla_{TEM}$, $bla_{SHV}$, and $bla_{CTX-M}$ genes was performed in 25 µL reaction mixtures containing 25 units/mL of Taq DNA polymerase, 200 µmol/L each of dATP, dGTP, dTTP, and dCTP, 0.2 µmol/L of each primer, 1.5 mmol/L $MgCl_2$, and 5 µL of DNA template[14]. The PCR products were analyzed by gel electrophoresis using 0.8% gel[15].

## RESULTS

Based on the demographic data of the enrolled patients[15], 25% were male and 45 (75%) were female. The age of the patients ranged from 12 to 67 years. All of the 60 *E. coli* isolates were found to be susceptible to doripenem (100%) and ertapenem (100%). High resistance rates to ampicillin (86%), cefotaxime (80%), and cefazolin (77%) were also found in the collected isolates (Table 1). Based on the CLSI confirmatory test, the frequency of ESBL-producing *E. coli* in KTPs was found to be 33.4%. Using the slide agglutination method, the most frequent serotypes were found to be serotype I (including: O126, O55 and O111; 50%), serotype II (O86, O127; 15%), serotype III (O44, O125, O128; 25%), and serotype VI (O120, O114; 10%). The genes most frequently found were $bla_{TEM}$ (55%), followed by $bla_{CTX-M}$ (51%) and $bla_{SHV}$ (41%).

**Table 1 Antimicrobial susceptibility patterns of *Escherichia coli* isolates from kidney transplant patients**

| Antibiotic | Susceptible (%) | Intermediate (%) | Resistant (%) |
|---|---|---|---|
| Ampicillin | 5 (8) | 1 (2) | 54 (90) |
| Amoxicillin-clavulanic acid | 28 (46) | 28 (46) | 23 (38) |
| Ampicillin-sulbactam | 26 (44) | 8 (12) | 26 (44) |
| Piperacillin-Tazobactam | 40 (67) | 6 (8) | 14 (24) |
| Cefazolin | 40 (67) | 8 (12) | 12 (20) |
| Cefepime | 27 (45) | 7 (12) | 25 (43) |
| Cefotaxime | 10 (17) | 1 (2) | 39 (65) |
| Doripenem | 60 (100) | 0 (0) | 0 (0) |
| Ertapenem | 60 (100) | 0 (0) | 0 (0) |
| Fosfomycin | 57 (95) | 2 (3) | 1 (1) |
| Imipenem | 57 (95) | 3 (5) | 0 (0) |
| Meropenem | 36 (60) | 10 (17) | 0 (0) |
| Amikacin | 40 (67) | 14 (25) | 14 (23) |
| Tobramycin | 41 (68) | 10 (17) | 9 (15) |
| Trimethoprim | 10 (17) | 13 (22) | 37 (61) |
| Nitrofurantoin | 48 (82) | 6 (8) | 6 (8) |
| Ciprofloxacin | 16 (27) | 4 (6) | 40 (67) |
| Gentamycin | 43 (71) | 6 (8) | 6 (8) |
| Cefpodoxime | 20 (34) | 2 (2) | 38 (64) |

## DISCUSSION

UTI is the main infectious complication in patients with kidney transplants. The high incidence of ESBL-producing *E. coli* among KTPs has been frequently reported[4]. In the current study, the frequency of ESBL-producing *E. coli* in KTPs was found to be 33.4%. A similar observation was noted by Linares *et al*[16], who reported that the incidence of ESBL-producing gram-negative bacteria in renal transplantation was 11.8%. Previous antibiotic therapy is an important risk factor for the development of ESBL-producing bacteria[17,18]. ESBL-producing *E. coli* infection is commonly associated with a significantly longer hospital stay and greater hospital charges[19].

According to the current study, high resistance rates to ampicillin (86%), cefotaxime (80%) and cefazolin (77%) were documented. Our results were comparable to a previous study that was conducted in Iran and reported a similar resistance rate to ampicillin[20].

In the current study, the most frequent ESBL genes were $bla_{TEM}$ (55%), followed by $bla_{CTX-M}$ (51%) and $bla_{SHV}$ (41%). In Portugal, studies from individual hospitals have reflected a common spread of $bla_{CTX-M}$ and $bla_{TEM}$[21]. Studies reporting different ESBL-producing bacteria are increasing among European countries[22]. A high prevalence of *E. coli* and *K. pneumoniae* isolates exhibiting two or three ESBL genes was also reported in a similar study from Iran[23]. The epidemiology of ESBL-producing bacteria is becoming more complex[24]. For example, *E. coli* harboring $bla_{CTX-M}$-15 and -14 have consistently been reported as the predominant ESBL types in clinical isolates from adult centers worldwide[25-27], yet a wide diversity of CTX-M enzymes was observed in children[28-30]. Moreover, it should be taken into consideration that bacterial isolates producing ESBLs are responsible for serious healthcare-related infections[31].

## CONCLUSION

In conclusion, the frequency of ESBL-producing *E. coli* in KTPs was found to be 33.4% in the current study. Molecular analysis showed that $bla_{TEM}$ was the most common ESBL encoding gene. The high resistance to β-lactams antibiotics (*i.e.*, ampicillin,

cefotaxime, and cefazolin) found in *E. coli* from KTPs with UTI remains a serious clinical challenge. Further efforts to control ESBL-producing *E. coli* should include the careful use of all antibiotics as well as barrier precautions to reduce spread.

## ARTICLE HIGHLIGHTS

### Research background

*Escherichia coli* (*E. coli*) isolates are the main pathogens in urinary tract infections (UTIs). Their effect is more important in kidney transplant patients (KTPs). Based on several studies and documents, the frequency of *E. coli* resistant to common drugs is increasing. Their resistance to antimicrobial drugs is mediated by different mechanisms such as producing extended-spectrum beta-lactamase (ESBLs). Therefore, UTIs caused by ESBL-producing *E. coli* in KTPs is an important challenge in healthcare settings.

### Research motivation

However, different studies have reported the frequency of ESBLs *E. coli* isolates from different origins in Iran, but there are few studies on their frequency and role in KTPs and their antimicrobial susceptibility profile.

### Research objectives

The aims of this study were: (1) To estimate the prevalence of ESBL-producing *E. coli* in KTPs; (2) To serotype the ESBL-producing *E. coli*; and (3) To identify the antibacterial susceptibility patterns of isolated bacteria in Tehran, Iran.

### Research methods

Bacterial culture and isolation based on standard bacteriologic methods were carried out. Antimicrobial susceptibility testing based on the Clinical Laboratory and Standards Institute was performed. The minimum inhibitory concentration was determined using Epsilon strips during the E-test. The frequency of genes responsible for ESBLs coding was assessed after DNA extraction and polymerase chain reaction. Statistical analysis of the data was performed.

### Research results

The most important findings were: (1) The frequency of ESBL-producing *E. coli* in KTPs was found to be 33.4%; (2) High resistance rates to ampicillin (86%) and cefotaxime (80%) were documented; (3) The most frequent serotype was serotype I (50%); (4) The most frequently found related gene was $bla_{TEM}$ (55%); and (5) All of the *E. coli* isolates were susceptible to doripenem and ertapenem.

### Research conclusions

Further efforts to control ESBL-producing *E. coli* isolates should include the careful use of all antibiotics as well as barrier precautions to reduce their spread.

### Research perspectives

More *E. coli* isolates from different parts of Iran should be obtained and their antimicrobial profiles evaluated. Also, the frequency of ESBLs production and the existence of other ESBLs genes such as *KPC* and *metalo-betalactamases* should be determined.

## REFERENCES


1 **Halaji M**, Shahidi S, Atapour A, Ataei B, Feizi A, Havaei SA. Characterization of Extended-Spectrum β-Lactamase-Producing Uropathogenic *Escherichia coli* Among Iranian Kidney Transplant Patients. *Infect Drug Resist* 2020; **13**: 1429-1437 [PMID: 32523361 DOI: 10.2147/IDR.S248572]

2 **Shams SF**, Eidgahi ES, Lotfi Z, Khaledi A, Shakeri S, Sheikhi M, Bahrami A. Urinary tract infections in kidney transplant recipients 1st year after transplantation. *J Res Med Sci* 2017; **22**: 20 [PMID: 28458711 DOI: 10.4103/1735-1995.200274]

3 **Abo Basha J**, Kiel M, Görlich D, Schütte-Nütgen K, Witten A, Pavenstädt H, Kahl BC, Dobrindt U, Reuter S. Phenotypic and Genotypic Characterization of *Escherichia coli* Causing Urinary Tract Infections in Kidney-Transplanted Patients. *J Clin Med* 2019; **8**: 988 [PMID: 31284699 DOI: 10.3390/jcm8070988]

4 **Golębiewska JE**, Krawczyk B, Wysocka M, Ewiak A, Komarnicka J, Bronk M, Rutkowski B, Dębska-

Ślizień A. Host and pathogen factors in Klebsiella pneumoniae upper urinary tract infections in renal transplant patients. *J Med Microbiol* 2019; **68**: 382-394 [PMID: 30747620 DOI: 10.1099/jmm.0.000942]

5 **Govindaswamy A**, Bajpai V, Khurana S, Aravinda A, Batra P, Malhotra R, Mathur P. Prevalence and characterization of beta-lactamase-producing *Escherichia coli* isolates from a tertiary care hospital in India. *J Lab Physicians* 2019; **11**: 123-127 [PMID: 31160850 DOI: 10.4103/JLP.JLP_122_18]

6 **Joshi P**, Khan ZA, Tandle R, Harshe A, Bhutada A, Gogavale S. Antibiotic Susceptibility Profile and Prevalence Pattern of Gram Negative Pathogens in Tertiary Care Hospital. *AJMPS* 2019; **7**: 1-9 [DOI: 10.9734/ajrimps/2019/v7i430125]

7 **Rameshkumar G**, Ramakrishnan R, Shivkumar C, Meenakshi R, Anitha V, Venugopal Reddy YC, Maneksha V. Prevalence and antibacterial resistance patterns of extended-spectrum beta-lactamase producing Gram-negative bacteria isolated from ocular infections. *Indian J Ophthalmol* 2016; **64**: 303-311 [PMID: 27221683 DOI: 10.4103/0301-4738.182943]

8 **Kuch A**, Zieniuk B, Żabicka D, Van de Velde S, Literacka E, Skoczyńska A, Hryniewicz W. Activity of temocillin against ESBL-, AmpC-, and/or KPC-producing Enterobacterales isolated in Poland. *Eur J Clin Microbiol Infect Dis* 2020; **39**: 1185-1191 [PMID: 32096107 DOI: 10.1007/s10096-020-03844-5]

9 **Dirar M**, Bilal N, Ibrahim ME, Hamid M. Resistance Patterns and Phenotypic Detection of β-lactamase Enzymes among Enterobacteriaceae Isolates from Referral Hospitals in Khartoum State, Sudan. *Cureus* 2020; **12**: e7260 [PMID: 32195070 DOI: 10.7759/cureus.7260]

10 **Clinical and Laboratory Standards Institute (CLSI)**. Performance Standards for Antimicrobial Susceptibility Testing. 29th ed. CLSI supplement M100. Wayne, PA: Clinical and Laboratory Standards Institute. 2019. Available from: https://shop.clsi.org/media/3226/m100-s29_definitions_correction_notice_2.pdf

11 **Sanchez DG**, de Melo FM, Savazzi EA, Stehling EG. Detection of different β-lactamases encoding genes, including bla NDM, and plasmid-mediated quinolone resistance genes in different water sources from Brazil. *Environ Monit Assess* 2018; **190**: 407 [PMID: 29909525 DOI: 10.1007/s10661-018-6801-5]

12 **Ori EL**, Takagi EH, Andrade TS, Miguel BT, Cergole-Novella MC, Guth BEC, Hernandes RT, Dias RCB, Pinheiro SRS, Camargo CH, Romero EC, Dos Santos LF. Diarrhoeagenic Escherichia coli and Escherichia albertii in Brazil: pathotypes and serotypes over a 6-year period of surveillance. *Epidemiol Infect* 2018; **147**: 1-9 [PMID: 30229714 DOI: 10.1017/S0950268818002595]

13 **Dilhari A**, Sampath A, Gunasekara C, Fernando N, Weerasekara D, Sissons C, McBain A, Weerasekera M. Evaluation of the impact of six different DNA extraction methods for the representation of the microbial community associated with human chronic wound infections using a gel-based DNA profiling method. *AMB Express* 2017; **7**: 179 [PMID: 28929383 DOI: 10.1186/s13568-017-0477-z]

14 **Haghighatpanah M**, Mozaffari Nejad AS, Mojtahedi A, Amirmozafari N, Zeighami H. Detection of extended-spectrum β-lactamase (ESBL) and plasmid-borne bla CTX-M and bla TEM genes among clinical strains of Escherichia coli isolated from patients in the north of Iran. *J Glob Antimicrob Resist* 2016; **7**: 110-113 [PMID: 27721192 DOI: 10.1016/j.jgar.2016.08.005]

15 **Singh V**, Wilks C, Reddy J, Granger J. Outpatient Urinary-Tract-Infection-Like Symptoms: Causative Microbial Survey Utilizing Multiplex Quantitative Polymerase Chain Reaction Methodology. *Adv Infect Dis* 2020; **10**: 26-36 [DOI: 10.4236/aid.2020.101003]

16 **Linares L**, Cervera C, Cofán F, Lizaso D, Marco F, Ricart MJ, Esforzado N, Oppenheimer F, Campistol JM, Moreno A. Risk factors for infection with extended-spectrum and AmpC beta-lactamase-producing gram-negative rods in renal transplantation. *Am J Transplant* 2008; **8**: 1000-1005 [PMID: 18727176 DOI: 10.1111/j.1600-6143.2008.02197.x]

17 **Zerr DM**, Miles-Jay A, Kronman MP, Zhou C, Adler AL, Haaland W, Weissman SJ, Elward A, Newland JG, Zaoutis T, Qin X. Previous Antibiotic Exposure Increases Risk of Infection with Extended-Spectrum-β-Lactamase- and AmpC-Producing Escherichia coli and Klebsiella pneumoniae in Pediatric Patients. *Antimicrob Agents Chemother* 2016; **60**: 4237-4243 [PMID: 27139486 DOI: 10.1128/AAC.00187-16]

18 **Biset S**, Moges F, Endalamaw D, Eshetie S. Multi-drug resistant and extended-spectrum β-lactamases producing bacterial uropathogens among pregnant women in Northwest Ethiopia. *Ann Clin Microbiol Antimicrob* 2020; **19**: 25 [PMID: 32493343 DOI: 10.1186/s12941-020-00365-z]

19 **Ho PL**, Yip KS, Chow KH, Lo JY, Que TL, Yuen KY. Antimicrobial resistance among uropathogens that cause acute uncomplicated cystitis in women in Hong Kong: a prospective multicenter study in 2006 to 2008. *Diagn Microbiol Infect Dis* 2010; **66**: 87-93 [PMID: 19446980 DOI: 10.1016/j.diagmicrobio.2009.03.027]

20 **Moini AS**, Soltani B, Taghavi Ardakani A, Moravveji A, Erami M, Haji Rezaei M, Namazi M. Multidrug-Resistant Escherichia coli and Klebsiella pneumoniae Isolated From Patients in Kashan, Iran. *Jundishapur J Microbiol* 2015; **8**: e27517 [PMID: 26587220 DOI: 10.5812/jjm.27517]

21 **Cantón R**, Novais A, Valverde A, Machado E, Peixe L, Baquero F, Coque TM. Prevalence and spread of extended-spectrum beta-lactamase-producing Enterobacteriaceae in Europe. *Clin Microbiol Infect* 2008; **14** Suppl 1: 144-153 [PMID: 18154538 DOI: 10.1111/j.1469-0691.2007.01850.x]

22 **Coque TM,** Baquero F, Canton R. Increasing prevalence of ESBL-producing Enterobacteriaceae in Europe. *Euro Surveill* 2008; 13: 19044 [PMID: 19021958]

23 **Mehrgan H**, Rahbar M, Arab-Halvaii Z. High prevalence of extended-spectrum beta-lactamase-producing Klebsiella pneumoniae in a tertiary care hospital in Tehran, Iran. *J Infect Dev Ctries* 2010; **4**: 132-138 [PMID: 20351452 DOI: 10.3855/jidc.488]

24 **Martinez P**, Garzón D, Mattar S. CTX-M-producing Escherichia coli and Klebsiella pneumoniae isolated from community-acquired urinary tract infections in Valledupar, Colombia. *Braz J Infect Dis* 2012; **16**: 420-425 [PMID: 22964287 DOI: 10.1016/j.bjid.2012.05.001]

25 **Pitout JD**, Nordmann P, Laupland KB, Poirel L. Emergence of Enterobacteriaceae producing extended-spectrum beta-lactamases (ESBLs) in the community. *J Antimicrob Chemother* 2005; **56**: 52-59 [PMID: 15917288 DOI: 10.1093/jac/dki166]

26 **Hawkey PM**. Prevalence and clonality of extended-spectrum beta-lactamases in Asia. *Clin Microbiol Infect* 2008; **14** Suppl 1: 159-165 [PMID: 18154540 DOI: 10.1111/j.1469-0691.2007.01855.x]

27 **Shu JC**, Chia JH, Kuo AJ, Su LH, Wu TL. A 7-year surveillance for ESBL-producing Escherichia coli and Klebsiella pneumoniae at a university hospital in Taiwan: the increase of CTX-M-15 in the ICU. *Epidemiol Infect* 2010; **138**: 253-263 [PMID: 19619387 DOI: 10.1017/S0950268809990409]

28 **Yu F**, Chen Q, Yu X, Li Q, Ding B, Yang L, Chen C, Qin Z, Parsons C, Zhang X, Huang J, Luo Y, Wang L, Pan J. High prevalence of extended-spectrum beta lactamases among Salmonella enterica Typhimurium isolates from pediatric patients with diarrhea in China. *PLoS One* 2011; **6**: e16801 [PMID: 21390297 DOI: 10.1371/journal.pone.0016801]

29 **Poirel L**, Ortiz de la Rosa JM, Richard A, Aires-de-Sousa M, Nordmann P. CTX-M-33, a CTX-M-15 derivative conferring reduced susceptibility to carbapenems. *Antimicrob Agents Chemother* 2019; **63**: e01515-19 [PMID: 31527021 DOI: 10.1128/AAC.01515-19]

30 **Díaz-Agero Pérez C**, López-Fresneña N, Rincon Carlavilla AL, Hernandez Garcia M, Ruiz-Garbajosa P, Aranaz-Andrés JM, Maechler F, Gastmeier P, Bonten MJM, Canton R. Local prevalence of extended-spectrum beta-lactamase (ESBL) producing *Enterobacteriaceae* intestinal carriers at admission and co-expression of ESBL and OXA-48 carbapenemase in *Klebsiella pneumoniae*: a prevalence survey in a Spanish University Hospital. *BMJ Open* 2019; **9**: e024879 [PMID: 30826764 DOI: 10.1136/bmjopen-2018-024879]

31 **Latour K**, Huang TD, Jans B, Berhin C, Bogaerts P, Noel A, Nonhoff C, Dodémont M, Denis O, Ieven M, Loens K, Schoevaerdts D, Catry B, Glupczynski Y. Prevalence of multidrug-resistant organisms in nursing homes in Belgium in 2015. *PLoS One* 2019; **14**: e0214327 [PMID: 30921364 DOI: 10.1371/journal.pone.0214327]

Published by **Baishideng Publishing Group Inc**
7041 Koll Center Parkway, Suite 160, Pleasanton, CA 94566, USA
**Telephone:** +1-925-3991568
**E-mail:** bpgoffice@wjgnet.com
**Help Desk:** https://www.f6publishing.com/helpdesk
https://www.wjgnet.com