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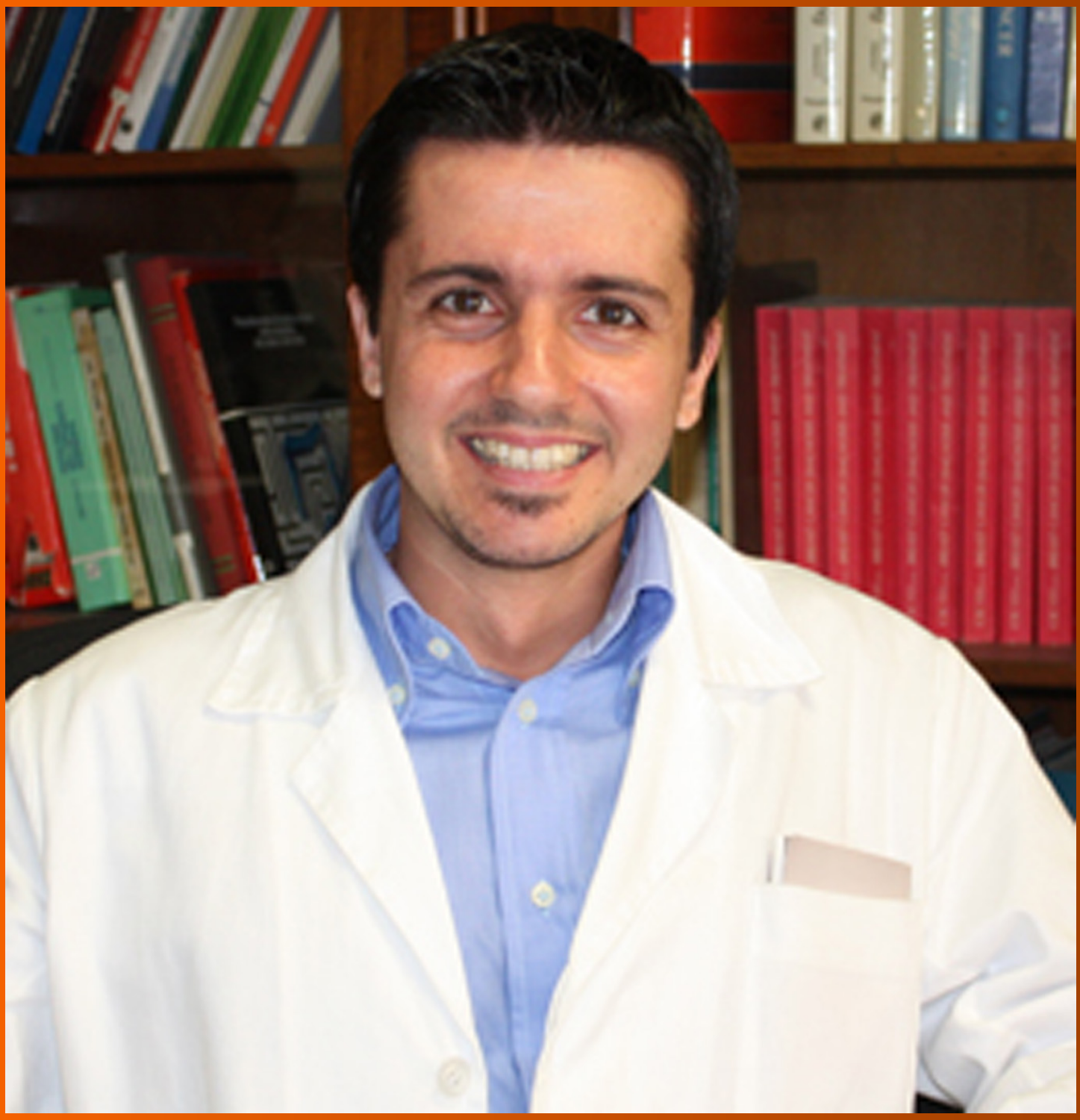

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Contents

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REVIEW

Contents

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REVIEW

Why the discovery of adherent-invasive *Escherichia coli* molecular markers is so challenging?

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Abstract

Adherent-invasive *Escherichia coli* (AIEC) strains have been extensively related to Crohn's disease (CD) etiopathogenesis. Higher AIEC prevalence in CD patients versus controls has been reported, and its mechanisms of pathogenicity have been linked to CD physiopathology. In CD, the therapeutic armamentarium remains limited and non-curative; hence, the necessity to better understand AIEC as a putative instigator or propagator of the disease is certain. Nonetheless, AIEC identification is currently challenging because it relies on phenotypic assays based on infected cell cultures which are highly time-consuming, laborious and non-standardizable. To address this issue, AIEC molecular mechanisms and virulence genes have been studied; however, a specific and widely distributed genetic AIEC marker is still missing. The finding of molecular tools to easily identify AIEC could be useful in the identification of AIEC carriers who could profit from personalized treatment. Also, it would significantly promote AIEC epidemiological studies. Here, we reviewed the existing data regarding AIEC genetics and presented those molecular markers that could assist with AIEC identification. Finally, we highlighted the problems behind the discovery of exclusive AIEC biomarkers and proposed strategies to facilitate the search of AIEC signature sequences.

Key words: Crohn's disease; Adherent-invasive *Escherichia coli*; Molecular markers; Genetics; Inflammatory bowel disease; Signature sequences

Core tip: In this review, we thoroughly review the approaches for deciphering adherent-invasive *Escherichia coli* (AIEC) genetics. The characteristics of putative AIEC molecular markers that could assist in AIEC identification are described. We then discuss several aspects that could explain the difficulty behind the discovery of suitable biomarkers and highlight the importance of standardizing AIEC protocols in order to increase the probability of finding these biomarkers. Finally, we point out new approaches for looking for signature sequences that need to take into account the AIEC

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phylogenetic origin and strain virulence under particular experimental conditions.

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INTRODUCTION

Non-pathogenic *Escherichia coli (E. coli)* strains are common colonizers of the mucus layer of the intestinal tract and have a mutualistic relationship with their hosts. However, some *E. coli* strains have evolved virulent behavior. Among those, strains belonging to the adherent-invasive *E. coli* (AIEC) pathovar are suggested to be of particular concern. AIEC isolates lack typical *E. coli* virulence factors but are phenotypically characterized by their capability to adhere to and invade intestinal epithelial cells (IECs), in addition to surviving and replicating inside macrophages without inducing host-cell death[1]. Using *in vitro* and *in vivo* studies, AIEC interactions with IECs have been described to occur through its binding to host receptors, which in turn, promotes intestinal epithelial permeability[2-5]. Additionally, in animal studies, induction of high levels of cytokine secretion and exacerbation of intestinal inflammation in susceptible hosts due to AIEC presence has been reported[6-8]. Since a high prevalence of AIEC has been depicted in the mucosa of Crohn's disease (CD) patients[1,9-16] and molecular mechanisms of AIEC virulence have been associated with disease pathogenesis[2,4-6,8,17-25], AIEC has been pointed out to take part in the complex multifactorial aetiology of CD.

It is of paramount importance to further decipher the role of AIEC in CD (such as disease specificity or association with active disease), AIEC host range and transmission paths in order to define measures of contamination risk and prevention and/or to provide personalized treatments for AIEC carriers. One reason for the lack of information in these aspects is due to the absence of an AIEC molecular biomarker. Its identification relies on phenotypic traits undergoing cell-culture infection assays, which are extremely time-consuming and hard to standardize. In this review, we aimed to provide a description of AIEC genetics based on the knowledge obtained by different approaches. Moreover, putative genetic/phenotypic markers for rapid AIEC identification have been gathered. We also researched putative reasons why finding AIEC molecular genetic signatures is challenging and discussed new strategies that could shed light on this field.

APPROACHES FOLLOWED TO DECIPHER AIEC GENETICS

Once Darfeuille-Michaud *et al*[1] defined the AIEC pathotype in 2004, a search for unique genes that could explain its phenotype began. Several approaches have been followed for deciphering AIEC genetics (gene prevalence, point mutations and gene expression) in which both known and novel genes have been studied.

First studies based on polymerase chain reaction (PCR)-based gene prevalence[11,26] indicated that AIEC strains did not harbor any particular genetic trait that could distinguish them from commensals and they did not commonly present virulence genes previously described in other *E. coli* pathotypes. In line with this observation, the first genome sequencing studies[27-30] together with the most recent genomic studies[31-36] demonstrated again that there was no gene strictly associated with the AIEC phenotype of the strains. Even though PCR-based and genomic studies focusing on gene content reported some genes to be more prevalent in AIEC versus non-AIEC strains (Invasion-related genes: *malX*[37], *pic*[38]; Capsule formation-related genes: *kpsMTII*[37]; Adhesion-related genes: *lpfA*[31], *papGII/III*[38]; Resistance-related genes: *gipA*[39], *ibeA*[40], *iss*[38]; Iron scavenging-related genes: *chuA*[13], *pduC*[31]; Toxin-related genes: *colV*[40], *vat*[38].). However, a low difference in AIEC/non-AIEC gene prevalence was reported for these genes (18%-34%; Table 1)[41,42], and only the *vat* gene was found to be differently distributed between AIEC and non-AIEC strains in other strain collections[32,43]. No confirmation of the findings in other studies has been obtained for 10/12 of these genes (*malX*[26,44], *kpsMTII*[16,26], *lpfA* and *gipA*[13], *chuA*[31,38], *pduC*[44], *ibeA*[37,38], *colV*[31], *pic*[37], *papGII/III*[16,37] and *iss*[31]). As a previous study pointed out[34], it is

likely that the associations described are phylogenetic in nature and do not reflect the pathogenic potential of the strains.

Controversial results on gene frequency may be explained by differential strain collections (origin of isolation, host and phylogenetic origin) and the amount of strains considered (Table 1 and Table 2). Our strain collection[37,38] is mainly composed of B2 strains, but for example, the collection of a previous study[31] is enriched in A and B1. As a consequence, the results of studies comparing unequal strains could be questioned. Such is the case of Desilets *et al*[32] who reported that B2-strains harbored three genomic regions that were absent in non-AIEC strains, but in the last group, all were non-clinical isolates, and only two B2 strains were considered. Since the AIEC pathotype is genetically highly diverse by phylogroup and invasive determinants, cross-validation of observations in a strain collection is strongly recommended.

Besides, it has been suggested that variations in the sequence of particular genes (*fimH, chiA* and *ompA*) may uncover AIEC virulence abilities[2,3,18]. For FimH, previous studies have found some polymorphisms conferring higher adhesion ability but they have not detected a variant more prevalent in AIEC than in non-AIEC isolates[13,32,34,38,44,45], yet one has hypothesised that gene expression might explain the phenotype[45]. Regarding OmpA, five amino acid variants (V114I, F131V, D132Y, T228N, and A276G) were described when AIEC reference strain LF82 and the commensal K-12 protein sequence were compared. In this study, Rolhion *et al*[3] suggested that the amino acid substitutions present in the LF82 protein sequence favors invasion. Likewise, for ChiA, five amino acid changes (Q362K, E370K, V378A, V388E, and E548V) were found located in a chitin binding domain of AIEC strain LF82 in comparison with K-12[18]. These differences in the amino acid sequence were thought to be responsible for the capability of the strain to adhere and invade IECs, as well as, to be a putative AIEC identification marker. Therefore, one of the studies conducted by our research group consisted of the examination of the protein sequences of ChiA, OmpA, OmpC, and OmpF in a large collection of strains[38,46]. In general, no relevant differences in the pathoadaptative mutations according to pathotype were reported; instead, most of them were related to phylogroup. Only one amino acid substitution in OmpA (A200V) and three in OmpC (S89N, V220I, and W231D) were associated with pathotype, but these genetic traits presented low specificity and sensibility as markers for AIEC screening. Despite no particular mutations in ChiA were associated with AIEC pathotype, we found that the LF82 ChiA sequence variant was mainly shared by AIEC strains. Nonetheless, it only comprised 35.5% of all AIEC strains. Thus, at this point, given that neither prevalence nor point mutations of the already described virulence genes (VGs) could uncover the basis of AIEC phenotype, identification of new genetic elements and application of novel techniques are required.

In 2010, the first AIEC genomes were sequenced, and since then many comparative genomics studies have been conducted in the attempt to elucidate the characteristics of the AIEC genome and to identify a genetic biomarker (Table 3)[47]. However, no gene or sequence exclusive to the AIEC pathotype has yet been identified. As a consequence, analysis of single nucleotide polymorphisms (SNPs) in the whole genome has attracted attention since it provided a novel approach to look for AIEC genetic markers. The first study using this methodology took place in 2015 in which only B2 strains were included[33]. Twenty-nine SNPs that could differentiate four AIEC together with 51 ExPEC strains from the commensal and other ExPEC strains were identified but no specific characteristic capable of distinguishing the AIEC pathotype was found[33]. This observation was in concordance with results from a study by O'Brien *et al*[34], who analyzed differences in base composition of genes among AIEC and non-AIEC strains from the same sequence type. No clustering of AIEC strains was observed. In contrast, the comparative genomics study of AIEC/non-AIEC strain pairs[36] revealed three SNPs [E3-E4_4.3(2), E3-E4_4.4 and E5-E6_3.16 = 3.22(2)] that resulted in differential nucleotide distribution between AIEC and non-AIEC strains in a larger strain collection (22 AIEC and 28 non-AIEC strains). However, there was no nucleotide only present in AIEC strains and absent in non-AIEC. Thus, this study corroborated the absence of AIEC-specific genetic markers widely distributed across all AIEC strains. In fact, the results obtained by analyzing gene prevalence and point mutations reinforce the idea that no particular VGs or pathoadaptative mutations described so far are specifically linked with the AIEC pathotype although, diverse genetic traits could lead to the same phenotype. However, studies reinforcing this hypothesis are absent, and a specific signature sequence of these strains remains to be elucidated.

In spite of the advances in the understanding of AIEC genetics, AIEC/non-AIEC differential gene expression has been scarcely studied[21,35,48]. Indeed, three earlier studies examined only LF82 against HS or K-12 gene expression. Furthermore, they studied only one gene during intramacrophage bacterial replication[21], seven genes in

Table 1 Phenotype, phylogroup and prevalence of virulence genes found to be more frequent in adherent-invasive *Escherichia coli* than non-adherent-invasive *Escherichia coli* strains in PCR-based and genomic studies

Virulence gene	Group of study (*n*)		Phylogroup (*n*)[1]					Prevalence (%)	
	AIEC	non-AIEC	A	B1	B2	D	Others	AIEC	non-AIEC
malX[37]	49	134[2]	39	19	98	19	8	71	47
kpsMTII[37]	49	134[2]	39	19	98	19	8	71	52
pduC[31]	24	25	14	16	10	9	0	50	20
lpfA[31]	24	25	14	16	10	9	0	71	20
lpfA + *gipA*[39]	35	103	Undetermined					31	0
chuA[13]	15	37	11	5	18	18	0	93	59
ibeA[40]	19	57	Undetermined					37	3
colV[40]	19	57	Undetermined					42	16
vat[38]	22	37	9	8	29	12	1	59	30
pic[38]	22	37	9	8	29	12	1	41	16
papGII/III[38]	22	37	9	8	29	12	1	18	0
iss[38]	22	37	9	8	29	12	1	32	11

[1]Based on house-keeping genes identified by triplex PCR[41] or by structure analysis included in the multilocus sequence typing analyses[42].
[2]Nineteen intestinal pathogenic *Escherichia coli* strains and 78 ExPEC strains isolated from animals and 37 human mucosal-associated non-AIEC strains. AIEC: Adherent-invasive *Escherichia coli*.

the presence of bile salts[48] or comparative transcriptomics while growing in Luria broth medium[35]. Our research contributed to these findings by studying outer membrane proteins (OMPs) gene expression in a collection of AIEC/non-AIEC strains[46]. We analyzed gene expression during bacterial intestinal epithelial cells (IEC) invasion. An increase in OMPs expression was reported in non-AIEC strains during IECs infection in comparison to the expression during growth in the supernatant of cell cultures, while AIEC strains only presented differences between conditions for *ompA* gene expression. Consequently, it is suggested that OMPs expression may participate in bacterial adhesion to IECs and intracellular persistence. Future work is required to confirm the implication of the differential expression in the AIEC phenotype by performing expression mutants and deciphering whether the differential expression is a trait common to all AIEC strains by studying the gene expression in a larger strain collection.

PUTATIVE BIOMARKERS TO ASSIST AIEC IDENTIFICATION

To date, eight genetic elements have been suggested as putative AIEC molecular markers (Table 4), however most of them presented either present low sensitivity or have been studied in a reduced number of strains. The putative biomarkers presented by Dogan *et al*[31] and Vazeille *et al*[39] were more prevalent in AIEC than in non-AIEC strains, nonetheless they were also present in non-AIEC strains (*pduC* and *lpfA*) (although in low percentages), or found only in a reduced number of AIEC strains (*lpfA* + *gipA*). As a consequence, the specificity values remained high, but the sensitivity values were low. The opposite occurred for the *chuA* gene[13]; in this case, it was present in nearly all of the AIEC strains and in more than 50% of non-AIEC strains and yielded a high sensitivity and high probability of false-positives (low specificity). Deshpande *et al*[33] discovered 29 SNPs that could differentiate a group of AIEC strains from a group of ExPEC and commensal strains (all from the B2 phylogroup), but they only studied four AIEC strains. Moreover, the three genomic regions described by Desilets *et al*[32] also raised interest. Nevertheless, it should be noted that only six non-AIEC strains were included, and AIEC strains were classified based only in the capacity to replicate within macrophages. Likewise, as only B2 strains were studied, the general utility of this approach for any putative AIEC strain remains to be determined.

Along this line, two additional markers that present either higher sensitivity or have been studied in a larger strain collection than the previous ones were presented[36,38]. On one hand, in a recent study we have deeply characterized

Table 2 Review of studies in which the prevalence of particular virulence genes has been examined according to the adherent-invasive *Escherichia coli* pathotype

Ref.	AIEC	Non-AIEC	Genes studied
Darfeuille-Michaud *et al*[1], 2004	26	0	*afaD, eae, ipaC, tia*
Martinez-Medina *et al*[11], 2009	22	38	*afa/draBC, bfpA, cdtB,cnf1, eae, eltA, est, fimAvMT78, fimH, hlyA, ibeA, ipaH, iucD, neuC, papC, pCDV432, sfa/focDE, stx1, stx2*
Martinez-Medina *et al*[26], 2009	27	59	*afa/draBC, bfpA, bmaE, cdtB, cnf1, cvaC, eae, eltA, est, fimA, fimAvMT78, fimH, focG, gafD, hlyA, ibeA ,ipaH, iroN, iucD, kpsMII, kpsMIII, malX, neuC, papC, papGI, papGII, papGIII alleles,pCDV432, sat,* ***sfa/focDE****, sfaS, stx1, stx2, traT, usp*
Martinez-Medina *et al*[37], 2011	49	134	*afa/draBC, astA, bmaE, chuA, cnf, csgA, cvaB, cvaC, eaI, eitA, eitC, etsB, etsC, fimC, focG, fyuA, gafD, gimB, hlyA, hlyF, hra, ibeA, iha, ireA, iroN, irp2, iss, iucD, iutA,* ***kpsMTII****,* ***malX****, mat, neuC, nfaE, ompA, ompT, papC, papEF, papGI, papGII, papGII/III, papGIII, pic, pks, sat, sfa/foc, sfaS, sitA, sitD (chr.), sitD (epis.), tia, traT, tsh, vat*
Chassaing *et al*[5], 2011	249		***lpfA***
Conte *et al*[16], 2014	27	0	*afa/draBC, aggR, cnf1, cvaC, fimH, focG,* ***fyuA****, gafD, hlyA,* ***ibeA****, iutA, kpsMT1, kpsMT5, kpsMTII, kpsMTIII, nfaE, pAA, PAI*[1]*, papA, papC, papEF, papG alleles, sfa/focDE, traT*
Vazeille *et al*[39], 2016	35	103	***lpfA + gipA***
Céspedes *et al*[13], 2017	15	37	*afa/draBC, aufA, cdtB,* ***chuA****, cnf1, cvaC, eaaA, eatA, ecNA144, espC, espP,* ***fhuD****, fimAvMT78 , fimH, gipA, hlyA, ibeA,* ***irp2****, neuC, papC, pet, pic, ratA, sat, sepA, sfa/focDE, sigA, tsh, vat*
Dogan *et al*[40], 2018	19	57	*afaC, chuA, cnf1,* ***colV****, focG, fyuA, gsp, hcp,* ***ibeA****, iss, kpsMII, lpfA, malX, papC, pduC, pmt1, ratA, sfaDE, traC*
Camprubí-Font *et al*[38], 2019	48	56	*afa/draBC, bmaE, csgA, fimC, focG, gafD, hra, iha, mat, nfaE, papC, papEF,* ***papGII/III****, papGI, papGII, papGIII, sfa/foc, sfaS, tsh, chuA, eitA, eitC, fyuA, ireA, iroN, irp2, iucD, iutA, sitA, sitD, (epis.), sitD (chr.),* ***iss****, neuC, kpsMTII, ompA, ompT, traT, astA, cnf, sat,* ***vat****, hlyA, hlyF, ibeA, gimB, tia, malX,* ***pic****, pks, eaI, cvaB, cvaC, etsB, etsC, lpfA141, lpfA154, fimH, chiA, astA, cnf, sat,* ***vat***
Camprubí-Font *et al*[46], 2019	13	30	*ompA, ompC, ompF*

Genes associated with pathotype or origin of isolation are highlighted in bold.
[1]Pathogenicity island described in a virulent uropathogen. AIEC: Adherent-invasive *Escherichia coli*; CD: Crohn's disease patients; UC: Ulcerative colitis patients.

genetically and phenotypically a collection of AIEC and non-AIEC strains isolated from the intestinal mucosa of humans[38]. Therein, AIEC screening could be assisted by the evaluation of two traits (the presence of *pic* gene and ampicillin resistance). Although these traits are not specific and widely distributed across the pathotype, *E. coli* strains that have resistance to ampicillin and harbor the *pic* gene present an 82% probability of being AIEC. Its major problem was a high rate of false-positives; thus, it could only be used as an initial screening tool, and AIEC strains predicted by this method should be further tested phenotypically. Besides, this marker has been only studied in a particular strain collection; therefore, further validation in external collections would be required. On the other hand, in contrast to previous studies seeking to find AIEC genetic markers, the genome of three strain pairs that could be considered clones but that differed in phenotype were compared[36]. Using this methodological approach, the combination of three point mutations (E3-E4_4.4, E5-

Table 3 Summary of the comparative genomics studies conducted in adherent-invasive *Escherichia coli* to date

Ref.	AIEC	Non-AIEC	Phylogroup	AIEC origin of isolation
Miquel *et al*[27], 2010	1	21[1]	AIEC: B2; Commensals: 4A, 2B1, 1B2; ExPEC: 2B1, 6B2, 3D, 3E	From an I-CD patient
Nash *et al*[28], 2010	2	10[1]	AIEC: B2; Commensals: 2A; ExPEC: 7B2, 1E	From I-CD patients
Dogan *et al*[31], 2014	24	25	14 strains from A phylogroup, 16 B1, 10 B2 and 9 D[2]	From I-CD patients and controls
Desilets *et al*[32], 2015	14[3]	6	AIEC: A: 1; B1: 1; B2: 10; D: 1; F: 1. non-AIEC: A: 2; B1: 2; B2: 2	From CD and UC patients[47]
Zhang *et al*[35], 2015	13	11	AIEC: 1A, 1B1, 4B2, 1D, 5 Unknown. non-AIEC: 3A, 8 Unknown	From CD and UC patients and non-CD subjects
Deshpande *et al*[33], 2015	4	1307[1]	All B2	From CD patients
O'Brien *et al*[34], 2015	11	30	All B2, ST95	From IBD patients and controls
Camprubí-Font *et al*[36], 2018	3	3	AIEC: 1 B1, 1 B2 and 1 D. Non-AIEC: 1 B1, 1 B2 and 1 D	From CD patients and controls

The strain collection examined according to pathotype and phylogroup is depicted. Adherent-invasive *Escherichia coli* origin of isolation and study observations are also presented.
[1]Include commensals and ExPEC.
[2]Human AIEC: 1A, 1B1, 1B2 and 1D; Murine AIEC: 1B1 and 1 B2; Dog AIEC: 2 B2; Human non-AIEC A phylogroup.
[3]Apart from LF82, UM146 and NRG857c the other strains were only assessed for intramacrophage replication in J774 cells. AIEC: Adherent-invasive *Escherichia coli;* CD: Crohn's disease; UC: Ulcerative colitis.

E6_3.16 = 3.22(2), and E5-E6_3.12) resulted in the prediction of AIEC phenotype with a sensitivity of 82%, a specificity of 86%, and an accuracy of 84%. So far, to our knowledge, this method is the best one out of the available methods. However, before drawing conclusions on whether a molecular marker is adequate to identify AIEC strains, we recommend performing additional analyses to confirm the specificity, sensitivity, and accuracy of this method. First, the results should be verified using a larger set of strains, including AIEC and non-AIEC strains from other geographical origins. Second, since AIEC strains present similar genetic traits as ExPEC strains[1,9,11], determining the specificity of the method with other *E. coli* pathotypes, in particular ExPEC strains, would also be required. Finally, if the results of the previously mentioned analysis confirmed the usefulness of the purposed method, testing the utility of the tool in clinical specimens (both fecal and tissue biopsies) should be considered.

POSSIBLE REASONS WHY THE SEARCH FOR AIEC MOLECULAR MARKERS IS CHALLENGING

Failure to detect a molecular property strictly associated with AIEC so far could be explained by: How AIEC might be emerged or the fact that the approaches used so far are not enough appropriate. Moreover, the lack of a standardized method for AIEC phenotypic characterization can add confusion in the search for distinctive traits and/or in its validation in external strains collections.

AIEC isolates by no means represent uniform populations[32,34,35]. This pathotype is highly diverse based on genetic and phenotypic characteristics such as virulence gene carriage or serotype. Even though most of them belong to the B2 phylogroup, they can comprise all the principal phylogenetic groups (A, B1, B2, D, and others)[9-11,49,50]. Moreover, they present genetic similarities with ExPEC strains[1,9,11]. Therefore, the AIEC phenotype might be driven by the combination of various virulence genes that do not necessarily need to be the same for each AIEC strain. Since different mechanisms are involved in the colonization of the epithelium by AIEC, the hypothesis considers that there is no key determinant in common for all the AIEC strains, and different ones can lead to the same phenotype gains plausibility. One study recently described that the genetics of one particular AIEC strain changes during host-to-host transmissions[51], resulting in strains with different phenotypes that compete with the parental strain and present a mobile element that is only

Table 4 Genetic elements more frequently found in strains from the adherent-invasive *Escherichia coli* pathotype and suggested as putative adherent-invasive *Escherichia coli* molecular markers

Marker	Group of study (*n*)		Prevalence (%)		Sensitivity (%)	Specificity (%)	Accuracy (%)
	AIEC	non-AIEC	AIEC	non-AIEC			
pduC[31][1]	24	25	50	20	50	80	65
lpfA[31][1]	24	25	71	20	71	80	75
29 SNPs[33][2]	4	1307	100	4	-	-	-
lpfA + *gipA*[39]	35	103	31	0	31	100	83
3 genomic regions[32][3]	14	6	79	0	79	100	85
chuA[13][4]	15	37	93	59	93	41	56
SNP algorithm[36]	22	29	-	-	82	86	84
pic + *ampR*[38]	22	27	86	33	86	67	75

[1]This strain collection was mainly formed by strains from A and B1 phylogroup (14 A, 16 B1, 10 B2 and 9D).
[2]Only B2 strains were included. In this case, the non-AIEC group included commensal and ExPEC strains.
[3]Only present in B2 AIEC strains. The strains' phylogroup were: AIEC: 1 A, 1 B1, 10 B2, 1 D and 1 F; non-AIEC: 2 A, 2 B1 and 2 B2.
[4]Strain collection with mainly B2 and D strains (11 A, 5 B1, 18 B2 and 18 D). AIEC: Adherent-invasive *Escherichia coli*.

maintained in specific conditions. Therefore, these finding indicate that this strain can easily adapt to specific environmental pressures genetically, making the search for biomarkers even more complex.

Moreover, differential gene expression may determine the phenotypic characteristics of AIEC strains. Indeed, this finding could explain why previous works have not found a gene or a point mutation that is widely distributed and specific to AIEC. So far, only two studies have described the transcriptome of AIEC[35,48]. A total of only three AIEC strains have been studied, and the selected experimental designs did not allow the best picture of the real expression profiles during AIEC gut colonization to be obtained. New experimental approaches directed at examining these elements under particular conditions in which AIEC isolates behave differently from other strains may help in finding molecular markers for AIEC detection that will probably be useful for clinical samples. Modulation of gene expression might be determined in various ways, such as DNA methylation or transposable elements. DNA methylation has been described to occur in bacteria in a manner that clonal bacterial populations can be split by switching among alternative DNA methylation patterns[52]. For instance, as studied in an uropathogenic *E. coli* strain, the Pap pilin causes variations in the phase by a mechanism which involves methylation[53]. Likewise, in terms of transposable elements, one study previously demonstrated that through constant macrophage exposure, a commensal *E. coli* strain can evolve into a pathogenic strain (such as being able to survive inside macrophages or escape) by the acquisition of transposable element insertion[54]. On the whole, epigenetics and transposable elements are unexplored in AIEC research and should be considered once looking for AIEC characteristic elements.

Finally, regardless of the above-mentioned possible reasons, once looking for AIEC biomarkers, the first question the scientists face is the standardization of the current AIEC identification method. The lack of uniformity among laboratories is very problematic since it can result in different classification assays, which may lead to incorrect associations between genetic and phenotypic features. The vast majority of studies have classified an isolate as AIEC by analyzing all of its phenotypic characteristics *in vitro;* nonetheless, some discrepancies exist in the protocols (Table 5) and the selected cell lines (Figure 1)[55-62]. Variances in the multiplicity of infection (MOI) and time of infection, in addition to incubation conditions occurred. In terms of invasion assays, while most analyses were performed at a MOI of 10 with an infection time of 3 h and subsequent 1-h incubation with gentamicin (100 μg/mL), others assessed the invasive capacity with a higher MOI (20 or 100), less time of infection (30 min or, 1 or 2 h) and different antibiotic concentrations (50 μg/mL or 3 mg/mL). Additionally, there is even more variability with the protocols used to determine the capacity of the strains to survive and replicate inside macrophages. In these cases, the highest discrepancy occurred with respect to the infection conditions since some performed a centrifugation step to facilitate bacterial intramacrophage uptake, whereas others did not. After this time of infection, non-phagocytosed bacteria were treated with antibiotics at different concentrations and different incubation times. The most common procedure included a first step of 1 h with higher antibiotic concentration (100 μg/mL) followed by a second step of 24 h incubation with

reduction in antibiotic concentration (15, 20, or 50 μg/mL) even though other studies performed only one incubation step that consisted of 1 or 24 h steps with the same concentration of antibiotic (20, 50, or 100 μg/mL or 3 mg/mL).

Moreover, the cell lines used to date (Figure 1) might not be the most appropriate considering that for instance, I-407 and Hep-2 originate from cervical and epithelial carcinomas of unknown origin, respectively, and both result from HeLa contamination. As an exception, Caco-2 and T84 are derived from colorectal carcinomas, but it is poorly defined how applicable they are for AIEC identification based on CD pathogenesis. Similarly, for intramacrophage survival, the cell lines mostly used are J774 which is derived from murine origin[1,9-13,15,16]. Some studies have started to use human macrophage-derived monocytes, THP-1 cells, but bacterial intramacrophage survival methods differ among them[34,62,63].

In view of the lack of standardization, adhesion and invasion indices in addition to the replication index of the strains are highly variable among research groups. Taking into account the indices of the LF82 AIEC strain, which is commonly used as control in these procedures, the adhesion index fluctuates between 4.8 and 62.8 bacteria/cell[1,18,34,39,45,62,64-66], the invasion index varies from 0.12% to 12.2%[1,9,16,34,39,62,65-73] and the intramacrophage survival and replication index ranges between 223.0% and 580.0%[1,9,16,21,34,62,66,74]. This finding is of particular concern, especially for those strains that present low indices, which are close to the threshold value that classifies the strain as adherent/invasive. In this case, one strain in one laboratory may be considered AIEC, while in another may be classified as non-AIEC. Therefore, there is the need to solve this discrepancy in order to regulate AIEC strain classification. Without consistency in the actual screening method, it is difficult to search for AIEC genetic differences as we might be using inaccurate isolates. Although hypothesis can be obtained in a particular strain collection, then in the process of validation it is complicated to obtain a good accuracy maybe due to different phenotypic characterization.

IS THE AIEC PHENOTYPE AN ACQUIRED TRAIT OF *ESCHERICHIA COLI* STRAINS FROM THE GUT?

By looking at recently published data, it becomes believable that the AIEC phenotype is not permanent, yet one might suspect that one *E. coli* can acquire the AIEC phenotype under particular conditions and inversely, one AIEC strain without specific triggers might turn to a non-AIEC strain or to some extent modify its virulence level. One observation in line with this hypothesis is the fact that very genetically close *E. coli* strains (identical pulsed field gel electrophoresis profiles) can be classified as either AIEC or non-AIEC[11,36]. Indicating that these strains have evolved to a pathogenic condition *via* nearly imperceptible genetic, transcriptomic, or epigenomic changes that may occur in particular cases. Furthermore, Elhenawy *et al*[51] recently demonstrated that one AIEC strain (NRG857c) evolved during host-to-host transmission in mice models, resulting in a diversified population of isolates with two predominant phenotypes: (1) Hypermotile isolates; and (2) Isolates with improved acetate utilization. The first phenotype was due to the presence of an insertion sequence upstream of the flagellar regulator *flhDC*, which resulted in hypermotile strains with enhanced IECs invasion. However, the presence of this insertion was reversible in the absence of host selection, suggesting that with the absence of particular conditions, the AIEC virulence may be altered. In the same way, Proença *et al*[54] observed that under continuous macrophage pressure, one commensal strain evolved to increased intracellular survival due to the incorporation of a transposable element insertion. Thus, their observations reinforce the hypothesis of intra-host *E. coli* evolution to an adherent invasive phenotype and the importance of conducting experiments simulating disease conditions as much as possible, since the AIEC marker may only be detected under selective pressure conditions.

Taking all of these outcomes into account, one may consider that AIEC strains originated from non-AIEC strains from the gut. For that reason, in the foreseeable future, other approaches beyond genes or SNPs prevalence should be analyzed when looking for AIEC molecular markers. These approaches include transcriptomics, epigenetics, and the study of AIEC under conditions in which they behave differently from other pathotypes, perhaps during interactions with host cells. Nowadays, two studies on transcriptomics[35,48] have been conducted. One study described findings in which the AIEC LF82 strain growing in contact with bile salts caused an increase in the expression of genes involved in ethanolamine utilization in comparison to K-12 and also demonstrated that AIEC strains grew more after incubation with minimum media with bile salts supplemented with ethanolamine than non-AIEC[48]. Therefore,

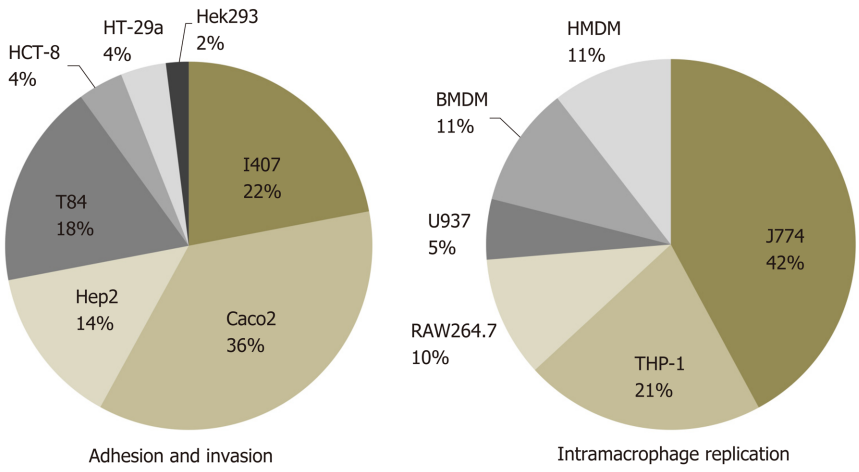

Figure 1 Review of cell lines used for adherent-invasive *Escherichia coli* identification. Analysis of the cell lines used for adhesion and invasion assays are based on 29 previously published works[1,9,11,13,14,16,18,21,31,34,39,43-45,55-62,64,66-68,70-72,74] while for intramacrophage replication 17 studies were considered[1,9,11,13,14,16,21,31,34,39,44,55,57,62,66-68,74].

reinforcing the idea that AIEC strains may adapt their metabolism according to gut conditions and that experimental methods need to be carefully considered when drawing conclusions about AIEC molecular traits. Nonetheless, the gene expression analysis of other non-AIEC and AIEC strains apart from K-12 and LF82 in the presence of bile salts has not been provided; thus, it is not possible to say that it is an AIEC-specific trait nor an adaptive method common among AIEC strains. Besides, Zhang *et al*[35] identified potential coding regions that could be applied as signature transcripts. Nevertheless, it is worth noting that they compared only one AIEC strain (LF82) with one commensal (HS) strain during growth in Luria broth. Thereby, the differences found between the strains could be strain-specific or perceptible due to the phylogenetic distance of the strains rather than to the AIEC phenotype. Given the extent of these studies and although there are some transcripts with a stimulating role in AIEC virulence, a candidate transcript suitable to be considered a universal and specific AIEC probe has not yet been determined. It is against this background that we encourage scientists to compare closely related strains and conduct the protocol in a more accurate environment in order to obtain a more a reliable interpretation of the gut context. For instance, previor to bacterial adhesion and invasion, bacteria need to cross the mucosal layer. As a consequence, an assay examining bacteria capacity to disrupt and translocate through the mucus should also be contemplated.

CONCLUSION

Although the factors that constitute an AIEC strain remain an enigma, the outcomes obtained by several lines of research over the last 15 years provide meaningful information on AIEC genetics. Gene prevalence, amino acid substitutions, and gene expression have been studied for both known and unknown genetic elements. In summary, research studies presented and discussed in this review demonstrate that AIEC is a diverse pathotype considering gene content and point mutations, and gene expression studies insinuated that the AIEC phenotype may be determined by particular differences in gene expression, but these need further verification using other AIEC strains.

The discovery of an AIEC biomarker would significantly ease further epidemiological studies in order to better determine AIEC prevalence and abundance and, discover environmental and animal reservoirs and transmission pathways in addition to facilitating clinical studies in CD patients. For example, studying the variations in abundance in relation to the state of the disease or in response to treatment might be useful. This type of biomarker would represent a rapid and cost-effective way to identify AIEC carriers, who could be treated with AIEC-directed therapies. So far, the diversity among AIEC strains challenges the correlation of individual virulence factors with pathotype in a way that is predictive. Moreover, AIEC classification as a non-AIEC from the gut that turns to pathogenic in particular conditions is gaining significance, but much remains to be learned about the host-pathogen interactions that govern AIEC infection biology. As a consequence, new

Table 5 Comparison of the principal experimental conditions of the protocols used to assess bacterial invasion to intestinal epithelial cells and survival and replication inside macrophages

MOI	Infection conditions	Incubation conditions	Ref.
Invasion assays			
10	30 min	3 h with amikacin 100 μg/mL	[13]
10	1 h	2 h with gentamicin 100 μg/mL	[70]
10 or 20	3 h	1 h with gentamicin 100 μg/mL	[1,9,11,14,16,31,34,47,56-60,63-65,71]
10	3 h	1 h with gentamicin 3 mg/mL	[72]
100	2 h	1 h with gentamicin 50 μg/mL	[54]
100	3 h	1 h with gentamicin 50 μg/mL	[62]
Survival and replication assays			
10	20 min	Media replacement with gentamicin 100 μg/mL for 40 min and media replacement with gentamicin 50 μg/mL for 24 h	[16]
10	2 h	Media replacement with amikacin 100 μg/mL for 3 and 24 h	[13]
10	2 h	Media replacement with gentamicin 100 μg/mL for 1 h and media replacement with gentamicin 20 μg/mL for 24 h	[1,34,58]
10 or 100	Centrifugation 10 min at 1000 g and incubation 10 min	Media replacement with gentamicin 100 μg/mL for 40 min and media replacement with gentamicin 20 μg/mL for 24 h	[11,47,66]
10	Centrifugation 5 min at 500 g and incubation 30 min	Media replacement with gentamicin 100 μg/mL for 2 h and media replacement with gentamicin 15 μg/mL for 24 h	[70]
20	2 h	Media replacement with gentamicin 100 μg/mL for 1 h and media replacement with gentamicin 20 μg/mL for 24 h	[9,31]
20	2 h	Media replacement with gentamicin 100 μg/mL for 1 and 24 h	[14]
20	2 h	Media replacement with gentamicin 3 mg/mL for 1 and 24 h	[72]
100	Centrifugation 10 min at 1000 g and incubation 10 min	Media replacement with gentamicin 20 μg/mL for 1 and 24 h	[21,59]
100	2 h	Media replacement with gentamicin 50 μg/mL for 1 and 24 h	[54]

approaches need to be performed in order to increase the probability of finding an AIEC molecular signature (these include but are not limited to SNPs in non-coding sequences, transcriptomics, metabolomics, and epigenomics). Nonetheless, all of these studies should be conducted using AIEC strains identified according to a standardized method, and the proposed methods should be tested in diverse strain collections from different geographical regions.

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Contents

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Contents

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FIELD OF VISION

Immunological aspects of COVID-19: What do we know?

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Abstract

The newly emerged coronavirus (severe acute respiratory syndrome coronavirus 2 SARS-CoV-2) and the disease that it causes coronavirus disease 2019 (COVID-19) have changed the world we know. Yet, the origin and evolution of SARS-CoV-2 remain mostly vague. Many virulence factors and immune mechanisms contribute to the deteriorating effects on the organism during SARS-CoV-2 infection. Both humoral and cellular immune responses are involved in the pathophysiology of the disease, where the principal and effective immune response towards viral infection is the cell-mediated immunity. The clinical picture of COVID-19, which includes immune memory and reinfection, remains unclear and unpredictable. However, many hopes are put in developing an effective vaccine against the virus, and different therapeutic options have been implemented to find effective, even though not specific, treatment to the disease. We can assume that the interaction between the SARS-CoV-2 virus and the individual's immune system determines the onset and development of the disease significantly.

Key Words: SARS-CoV-2; COVID-19; Immune memory; Anti-SARS-CoV-2 antibodies; COVID-19 treatment; Plasma therapy

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Core Tip: Since the coronavirus disease 2019 (COVID-19) results from the interaction between the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus and the individual's immune system, we can assume that its onset and development significantly depend on this communication. Immunological aspects of the disease reflect the importance of the immune system to inhibit the viral factors and to control and regulate the pathophysiological processes during SARS-CoV-2 infection. Moreover, immune-mediated and humoral immune responses, immune memory, the cytokine storm, and neuroendocrine-immune regulation are critical factors that can determine the prognosis and outcome for patients. Now, the science is directed to acquiring new data on the immunology, including immune memory against the virus, the development of new technologies for the detection of infection and effective vaccines. However, much more information remains unclear than verified knowledge of the SARS-CoV-2 virus and COVID-19.

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EPIDEMIOLOGY OF SEVERE ACUTE RESPIRATORY SYNDROME CORONAVIRUS 2 INFECTION

The novel coronavirus (formerly called HCoV-19) is a new coronavirus in humans that emerged at the end of 2019 (December) in Wuhan, China. Later, it received the name the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) that stands for severe acute respiratory syndrome coronavirus 2. Now it is the cause of the current pandemic[1]. Not surprisingly, coronaviruses (CoV) were intensively studied for the last decade, especially during the last months of the current pandemic, but not enough information is elucidated for them. It is known that CoV are zoonotic, single-stranded ribonucleic acid (RNA) viruses that cause a wide range of symptoms. The latter include those from common cold to more severe respiratory complaints as well as gastroenteric, hepatic, and neurological symptoms[2]. Except SARS-CoV-2, there are six other reported human coronavirus members. These are HCoV-OC43, HCoV229E, SARS-CoV, HCoV-HKU1, Middle East respiratory syndrome corona virus (MERS-CoV), and HCoVNL63[3,4]. Over the last twenty years, CoV have caused two significant epidemics: SARS[5] and MERS[6].

The positive-sense single-stranded RNA of SARS-CoV-2 is enveloped in a lipid bilayer. The virus belongs to the genus *Betacoronavirus* and family *Coronaviridae*[7]. Yet, the origin and evolution of SARS-CoV-2 remain vague. Furthermore, SARS-CoV-2-related viruses were found in Malayan pangolins (*Manis javanica*), as several recent studies showed. These data provided new insights into the evolution and host distribution of these SARS-CoV-2-related viruses[8,9].

Coronavirus disease 2019 (COVID-19) is the illness associated with SARS-CoV-2 infection. The clinical syndrome is characterized by variable symptoms, ranging from mild upper respiratory symptoms to severe interstitial pneumonia and acute respiratory distress syndrome (ARDS)[10,11].

SARS-CoV-2 and SARS-CoV and MERS-CoV belong to the same *Betacoronavirus* genus, and they share about 80% nucleotide identity. However, despite the close relation between SARS-CoV and SARS-CoV-2, the latter seems to cause milder infections[7]. Moreover, SARS and MERS were characterized mainly with nosocomial spread, whereas SARS-CoV-2 has community transmission[12].

Regarding clinical features, COVID-19 seems similar to SARS; however, it is considered to be less lethal than MERS, which differs from the other two CoV in terms of both phylogenetic and pathogenetic features. Due to the less severe clinical picture, COVID-19 can spread in the community more easily than MERS and SARS, which is reported in the nosocomial settings[13-15].

The spread of COVID-19 is rapid, which is somehow expected because the transfer is carried out by close contact and droplets[16]. However, there is scarce evidence to suggest airborne transmission, as very minimal to no viral RNA was detected in airborne samples, and no viral RNA was found in urine or serum samples of positive

patients[17].

Under the experimental circumstances tested, the stability of SARS-CoV-2 is similar to that of SARS-CoV-1. This indicates that their different epidemiologic features are probably due to other factors, such as high viral concentrations in the upper airways and the potential asymptomatic spread of SARS-CoV-2[18,19]. Also, the aerosol and fomite transmission of SARS-CoV-2 is likely since the virus can remain infectious depending on the inoculum shed. It was shown that the virus is viable in aerosols for hours and on surfaces up to days. These findings mirror those of SARS-CoV-1, in which both the nosocomial and super-spreading transmissions were observed. All of these characteristics provide information for pandemic mitigation efforts[20]. In summary, current evidence state that the COVID-19 virus is primarily transferred between people through respiratory droplets and contact routes[21]. Transmission due to droplets is documented in close contact (considered within 1-1.5 m) with a symptomatic person (*e.g.*, coughing or sneezing). The risk is estimated by the exposition of mucosae (mouth and nose) or conjunctiva (eyes) to potentially infective respiratory droplets. Fomites in the immediate environment around the infected person can also lead to transmission[22]. Therefore, the virus can be spread directly by infected people and indirectly through surfaces or objects used by the infected person[22].

Coronavirus is one of the significant pathogens that targets the human respiratory system primarily[10]. SARS-CoV-2 was found to have higher rates of transmissibility and therefore pandemic risk than SARS-CoV, as the current effective reproductive number (R) is 2.9, which is much higher than the R of SARS (1.77). Several COVID-19 studies have estimated the basic reproduction number ranges between 2.6 to 4.71[23]. Nevertheless, the Chinese health authorities have documented average incubation of 7 d (2-14 d)[24]. However, the case fatality rate (CFR) of any disease is not fixed and varies with location and time. It was shown that CFR of COVID-19 varies widely between countries ranging from 0.2% in Germany to 7.7 % in Italy[25]. The differences in the CFR might be due to various factors. On one hand, the greater the number of performed polymerase chain reaction tests, the less the CFR will be. On the other hand, underlying conditions and comorbidities of the patients should also be taken into account - has a patient died because of COVID-19 or as a result of an exacerbation of a chronic disease/or another sudden acute medical condition, such as stroke and heart failure, but with a positive polymerase chain reaction for SARS-CoV-2 RNA.

SARS-CoV-2 proved to be a great challenge. Adapted to the human body, it spread around the world in a short time. The air-drop mechanism is straightforward to implement. The natural spread of SARS-CoV-2 is due to the mechanism and the fact that in about 85% of patients, COVID-19 occurs in a mild form. Patients with a mild form do not seek medical help. Usually, they treat themselves and infect their contacts[26]. They do not limit themselves to their home, but travel, work, and are in contact with many people. It has been shown that asymptomatic individuals can be contagious and secrete SARS-CoV-2. This further facilitates the spread of COVID-19. From an epidemiological point of view, mild and asymptomatic infections are hazardous because people have no idea that they are contagious. They do not seek medical help, they are not examined, and by contact, infect others. This requires a strict search and examination of the contacts of proven individuals with COVID-19 for the detection of asymptomatic infectious.

SARS-COV-2: VIRULENCE FACTORS AND PATHOLOGICAL EFFECTS

As already mentioned above, COVID-19 is an infection that has shortly become a health problem of global concern. Although COVID-19 is not the first outbreak of a coronaviral disease, neither SARS nor MERS led to such a high number of cases worldwide. This suggests that SARS-CoV-2 is highly contagious and much more virulent than both SARS-CoV and MERS-CoV. The continuous expansion of COVID-19 could be explained with the viral-specific characteristics and virulence factors[27]. CoV are enveloped viruses, named for the spikes on their surface that resemble a crown. Their genome is organized in a positive single-stranded RNA. CoV are divided into four genera: α, β, γ, and δ. Along with SARS-CoV and MERS-CoV, the current coronavirus SARS-CoV-2 is classified as a β-coronavirus. Its genome sequence is about 88% identical to that of two bat-derived SARS-like viruses, and about 80% and 50% identical to the genome sequences of SARS-CoV and MERS-CoV, respectively[28]. Mutations and recombination of the viral genome frequently occur due to error-prone RNA-dependent-RNA polymerases of the CoV. These events are closely related to

viral adaptation[27]. CoV proteins include structural proteins: Spike (S), envelope (E), nucleocapsid (N), and membrane (M) proteins and some proteins with unknown function. S protein is a glycoprotein essential for viral entry by attachment and fusion to the cellular membrane (Figure 1). It is the main presented antigen on the viral surface and a target of neutralizing antibodies formed during the humoral immune response to the virus[28].

SARS-CoV and SARS-CoV-2 enter the host cell by binding to the angiotensin-converting enzyme 2 (ACE-2) receptor, while the dipeptidyl peptidase 4 receptor is required for MERS-CoV entry. After the virus enters the cell, viral RNA is released and involved in consecutive processes of new viral particle formation, which are then released[29]. ACE-2 receptors are mainly expressed in the vascular epithelium, renal tubular epithelium, and Leydig cells in the testes. For this reason, SARS-CoV might lead to hypogonadism and harm male fertility. In the respiratory system, SARS-CoV-2 enters the alveoli by binding to the ACE-2 receptors, predominantly expanded on the type II pneumocytes[30]. Once infected, type II pneumocytes are destroyed, and surfactant production is reduced. Macrophages are then recruited to destroy the damaged tissue of the lungs. Macrophages secrete interleukin (IL)-1, IL-6, and tumor necrosis factor alpha (TNFα), which cause vasodilatation and high temperature and enhance neutrophils and lymphocytes migration to the affected area. Alveoli edema causes respiratory failure due to blood-gas exchange disturbance. Nonstructural proteins might affect the innate immune response of the host and play a crucial role in the viral virulence and pathophysiology of SARS-CoV-2 infection[30].

In vitro and *ex vivo* examinations compared the viral tropism of SARS-CoV-2 with that of SARS-CoV, MERS-CoV, and 2009 pandemic influenza H1N1. It showed more extensive infection of bronchial epithelium, ciliated cells, and goblet cells with SARS-CoV-2 than with the other viruses[31]. A robust replication in human bronchus was observed, although ACE-2 receptor expression was relatively low in comparison with the lung parenchyma. However, the endothelium of the blood vessels was not found to be infected[32].

SARS-CoV-2 could also be detected in tears, anal swabs, and stool specimens. Moreover, infection and productive replication of the virus were observed in the conjunctiva and colorectal carcinoma cell lines. These findings suggest that conjunctiva and the bronchus epithelium might also be portals of infection and also poses the possibility of fecal-oral transmission. Various routes of viral transmission would explain the extensive spread of the SARS-CoV-2 throughout the whole world, causing a pandemic[32].

CoV have developed many mechanisms to avoid the immune system, which allow them to better survive in host cells. One of the multiple strategies is the forming of double-membrane vesicles that lack pattern recognition receptors when shedding, thus avoiding the recognition of their evolutionary old and conservative pathogen-associated molecular patterns, such as double-stranded RNA[33]. Moreover, studies in mice showed that SARS-CoV and MERS-CoV infection might inhibit the most potent anti-viral molecules, such as interferons (IFN)-I (IFN-α and IFN-β)[34,35]. The described mechanisms behind this inhibition in MERS-CoV include blocking of melanoma differentiation-associated protein 5, inhibiting the nuclear transport of IFN regulatory factor 3, suppressing the antigen presentation[29], *etc*. Therefore, the ability of SARS-CoV-2 to avoid the action of the immune system is a critical factor in the treatment of current infection and the development of specific drugs.

HUMORAL AND CELLULAR IMMUNE RESPONSE – DANCING ON THE FIRE

The presentation of virus antigens stimulates the humoral and cellular immune response, which are exerted by virus-specific B and T lymphocytes, respectively. Like common acute viral infections, the antibody production against SARS-CoV viruses has a typical immunoglobulin (Ig)M and IgG pattern. SARS-specific IgM antibodies may disappear at the end of the 3rd mo. In contrast, the IgG antibody may persist longer, indicating that IgG antibodies are likely to be protective[29]. SARS-specific IgG antibodies are predominantly against S- and N-proteins.

Immunological follow-up revealed a progressive increase in plasma SARS-CoV-2-specific IgM and IgG antibodies from 1st to 3rd wk[36].

Patients with COVID-19 may worsen clinically after some days of illness (especially around day 9), which is accompanied by an increase in the pro-inflammatory response, which often leads to admission to intensive care units and the need for

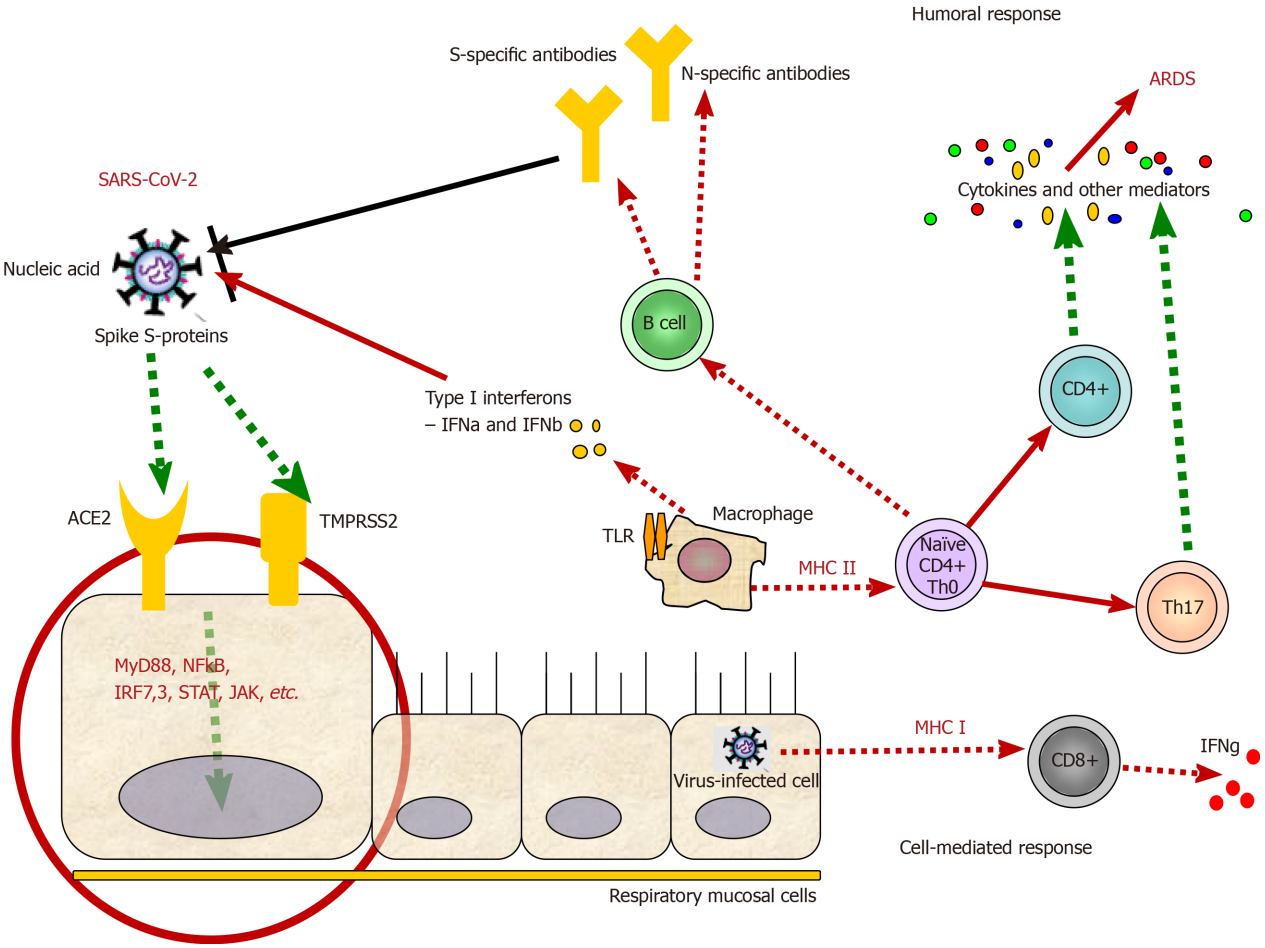

Figure 1 Virulence factors and immune mechanisms during severe acute respiratory syndrome coronavirus 2 infection. After the recognition of the virus by angiotensin-converting enzyme 2 and/or TMPRSS2 receptors, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) internalizes in the host cell. *Via* several secondary messengers, the respiratory mucosal cell is stimulated to secrete cytokines and present viral antigens *via* major histocompatibility complex (human leukocyte antigen) class II molecules to cytotoxic CD8+ T cells, which further secrete cytokines, such as interferon gamma. Additionally, natural killer cells (not shown) also contribute to the killing of infected host cells along with T cytotoxic cells as a part of cellular immunity against every viral infection. Antigen-presenting cells, such as macrophages and dendritic cells, can present viral particles *via* human leukocyte antigen class II molecules and prime CD4+ T helper cells, which further differentiate to different Th cells, such as Th17 cells (showed), which secrete a vast majority of cytokines, leading to cytokines storms and acute respiratory distress syndrome. Th cells stimulate B cells to produce antibodies against some SARS-CoV-2 antigens, part of the humoral immunity against the virus. Viral replication and shedding are not shown. ARDS: Acute respiratory distress syndrome; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; ACE2: Angiotensin-converting enzyme 2.

supportive mechanical ventilation. This secondary deterioration is reminiscent of SARS-CoV, in which 80% of patients with SARS-CoV develop acute respiratory disease at the time of anti-viral IgG seroconversion[37]. IgG seroconversion is associated with two points in the immune response - the disappearance of the virus and the appearance of IgG antibodies. IgM antibodies may persist for some time together with IgG. Moreover, patients who produce anti-S-neutralizing antibodies at the onset of the disease have a higher risk of death. The mechanisms behind these observations are not clear, but it is assumed that excessive complement activation may play a role. In addition, a phenomenon called antibody-mediated enhancement of viral infection may be responsible for persistent viral load and subsequently cause a direct or indirect effect on ACE-2 activity in the lung[37] and eventually death.

Compared to humoral responses, there are more studies on cellular immunity in coronavirus infection, which is not surprising, taking into account that the principal and effective immune response towards viral infection is cell-mediated immunity.

A clinical case of mild SARS-2-CoV infection and favorable outcome for the patient revealed that the antibody-producing immune cells, defined by the expression of CD3-CD19+CD27^{hi}CD38^{hi} and CD4+CXCR5+ICOS+PD-1+ Th follicular cells, appear in the blood during viral clearance (day 7; 1.48%) and peak on day 8 (6.91%); and on day 7 (1.98%), increasing on day 8 (3.25%) and day 9 (4.46%), respectively. The peak of both cell subpopulations was significantly higher in the COVID-19 patient than in healthy controls and persisted during convalescence[38]. Cytotoxic T cells also increased rapidly

from day 7 (3.57%) to day 8 (5.32%) and day 9 (11.8%), followed by a decrease on day 20, respectively. Besides, the incidence of CD38+human leukocyte antigen (HLA)-DR+CD8+ T cells was significantly higher in this patient than in healthy subjects (1.47% ± 0.50%)[37]. We have to emphasize once again that activated cytotoxic T cells, representatives of adaptive or so-called specific immunity, along with natural killer (NK) cells, part of innate immunity, are critical players in the cell-mediated immune response that accompanies any viral infection.

Helper CD4+ T cells simultaneously expressing CD38 and HLA-DR increased between day 7 (0.55%) and day 9 (3.33%) in this patient compared to healthy donors (0,63% ± 0.28%), although being in lower percentages than for CD8+ T cells. CD38+HLA-DR+CD8+ T cells produce about 34%-54% more granzymes A and B and perforin. The appearance and rapid increase of activated CD38+HLA-DR+ T cells, especially CD8+ T cells, on day 7-9 precede the disappearance of symptoms[37].

Analysis of CD16+CD14+ mononuclear cells, which are associated with the immunopathology of COVID-19, showed a lower frequency of these cells in the blood of this patient on days 7, 8, and 9 (1.29%, 0.43%, and 1.47%, respectively), compared to healthy control donors (9.03% ± 4.39%), probably indicative of their entry from the blood to the site of infection. No differences were found in activated HLA-DR+CD3-CD56+ NK cells during infection and compared to healthy levels[37].

However, recent data conclude that even reduced in numbers, Th and Tc cells are overactivated in patients infected with SARS-CoV-2[38]. This significant reduction is also observed in the acute-phase response in COVID-19 patients. However, once presented in the organism, CD4+ and CD8 + anti-virus memory T cells persist in the bloodstream of recovered patients for up to 4 years, even in the absence of viral antigens[39]. Moreover, other studies detected SARS-CoV-S protein-specific memory T memory cells 4 years after the patients` infection[40] and MERS-CoV-specific CD8+ T cells in mice, mainly involved in viral clearance[41]. These findings are a reasonable basis for designing effective vaccines against SARS-CoV-2.

Th17 cell responses are also involved in the immune pathogenesis of COVID-19, mainly with the secretion of various cytokines, such as IL-17, granulocyte-macrophage colony-stimulating factor, IL-21, and IL-22[42]. IL-1b and TNFα, as promoters of human Th17 cell differentiation, are also involved in stimulating vascular permeability and leakage. Nevertheless, it was shown that patients with a severe form of COVID-19 had increased levels of CCR+ Th17 cells[39]. This may suggest that Th17 cells and their cytokines are also involved in the cytokine storm. Besides, elevated Th17 and Th1 cell responses were also described in MERS-CoV and SARS-CoV patients[43,44]. These results support the hypothesis that enhanced IL-17-related pathways, including higher IL-17 but lower IFNγ and IFNα, are associated with worse outcomes for the patients[39].

As we have some proof of the contributing role of Th17 cells to the cytokine storm, Th17 cells may likely promote pulmonary edema, tissue damage, and lung failure. In line with this, targeting Th17 cells may be beneficial for some patients with COVID, especially in those with a dominant Th17 immune profile[43,45].

Some of the immunological responses that are accompanying the SARS-CoV-2 infection are presented in Figure 1.

CLINICAL MANIFESTATIONS OF COVID–19

A symptomatic COVID-19 case is defined as an infected person with a clinical picture suggestive of COVID-19. On the other hand, an asymptomatic case is an infected person who has not developed any signs or symptoms of COVID-19[46].

The incubation period of the disease is 5-6 d on average but can be up to 14 d. Critical epidemiological and immunological aspects of the disease are that an infected person may be contagious 1-3 d before the onset of the symptoms[47]. About 40% of COVID-19 patients experience mild clinical course, and another 40% present with moderate disease. Severe disease that requires oxygen support is observed in about 15% of the patients. Five percent of the infected ones develop a severe disease that progresses to respiratory failure (ARDS) but also sepsis and septic shock, thromboembolism, multiorgan failure, including acute kidney, and cardiac injury[48]. Advanced age, smoking, as well as comorbidities such as diabetes, hypertension, cardiac and chronic lung disease, cerebrovascular diseases, immunosuppression, and cancer have been reported to predispose to a severe course of COVID-19. Children and infants usually experience mild disease or asymptomatic infection[49].

There are no specific clinical symptoms of COVID-19 that can be taken as a reliable pathognomonic sign. Patients most commonly present with fever, cough (usually dry),

fatigue, and anorexia. Dyspnea is usually seen in severe cases. Other nonspecific symptoms include sore throat, nasal congestion, headache, nausea, as well as diarrhea and vomiting. Loss of smell and taste have also been reported. In older people, some atypical symptoms such as reduced alertness and/or mobility and delirium might be seen[50,51].

COVID-19 may also present with neurological and mental manifestations, including delirium or encephalopathy, agitation, stroke, meningoencephalitis, anxiety, depression, and sleep problems. In many cases, some of these neurological manifestations have been documented without respiratory symptoms[52,53].

A retrospective study of SARS-CoV-2 infected patients showed mild leucopenia in mild cases. Mild to moderate leukocytosis was observed in severe cases with significantly high neutrophil and significantly low lymphocyte count. In patients with a severe course of the disease, significantly high alanine aminotransferase and aspartate aminotransferase were observed, as well as hypoalbuminemia, elevated concentration of C-reactive protein, lactate dehydrogenase, ferritin, and D-dimer. These laboratory changes are a result of an acute and severe inflammatory response[50].

Research on cellular immunity in SARS-CoV-2 positive patients showed a significant reduction in CD4+ and CD8+ T lymphocytes[29]. A more significant decrease in T cells was observed in severe cases as well as an elevation of the concentration of serum cytokines (IL-2, IL-6, IL-10, and TNFα). Elevation of IL-6 concentrations was also found in moderate cases[50]. On that basis, the cytokine storm is a crucial factor for the clinical course of COVID-19 and the disease severity.

A common complication of SARS-CoV-2 infection is the development of ARDS. The latter is assumed as the leading cause of death in patients with COVID-19, especially among those with underlying diseases and conditions, evaluated as risk factors, smokers, and older age. ARDS is a result of the cytokine storm - an immunopathological event that leads to an uncontrolled systemic inflammatory response from the release of pro-inflammatory cytokines IFNα, IFNγ, IL-1β IL-3, IL-6, IL-12, IL-18, TNFα, and chemokines by the immune cells during SARS-CoV infection[29]. These biologically active substances seriously damage the lung parenchyma and lead to respiratory failure. This severe acute hypoxic status is accompanied by increased pulmonary capillary permeability and alveolar epithelial cell damage[51]. TNFα and IL-6 production in COVID-19 follows a different pattern than the pattern in bacterial sepsis or influenza. Furthermore, it was shown that blocking IL-6 by Tocilizumab restored partially HLA-DR expression and increased the number of circulating lymphocytes[51]. The immune mechanisms in COVID-19 are characterized by two main pathways, IL-6 or IL-1b-driven immune hyperactivation, leading to macrophages activations syndrome (MAS) or immune dysregulation (Figure 2).

The initiation of pneumonia in COVID-19 includes intense antigen presentation, accompanied by elevated C-reactive protein (CRP), D-dimer, and liver aminotransferases plus infiltrates in the lungs, whereas severe respiratory failure displays either MAS or deficient HLA-DR expression and profound depletion of Th, B cells, and NK cells[51]. In such a way, during ARDS, CRP, D-Dimer, and liver transaminases are further increased, leading to permanent pathological changes.

The described cytokine storm violently attacks not only the lungs but the organs of every system in the organism, causing multiple organ failure that leads to death in severe cases of SARS-CoV-2, SARS-CoV, and MERS-CoV infection[29].

Affecting the whole organism, the cytokine storm is the primary mechanism that induces disseminated intravascular coagulation (DIC). Pro-inflammatory cytokines TNFα and IL-1 suppress the endogenous anticoagulation. Inflammation damages the endothelium and leads to the release of tissue plasminogen activator, which could explain the elevation of the D-dimer and fibrin degradation products[54]. In summary, the accumulated data showed that COVID-19 is associated with a hypercoagulable state with increased risk of thromboembolic complications.

However, ARDS is assumed as the leading cause of death in COVID-19. During the early stages of the outbreak, it was reported that of the 41 SARS-CoV-2 infected patients admitted, six died of ARDS[48]. ARDS remains the most common immunopathological event for SARS-CoV-2, SARS-CoV, and MERS-CoV infections. One of the primary mechanisms for ARDS is the cytokine storm. The latter is a uncontrolled systemic inflammatory reaction resulting from the release of large amounts of pro-inflammatory cytokines (IFN-α, IFN-γ, IL-1β, IL-6, IL-12, IL-18, IL-33, TNF-α, TGFβ, *etc*.) and chemokines (CCL2, CCL3, CCL5, CXCL8, CXCL9, CXCL10, *etc*.) from immune effector cells during many viral infection[48,55-57]. Similar to SARS-CoV, individuals with severe MERS-CoV infection show elevated serum levels of IL-6, IFN-α, CCL5, CXCL8, and CXCL-10 compared to those with mild or moderate disease[37]. The cytokine storm causes ARDS and multi-/multiorgan failure, leading to

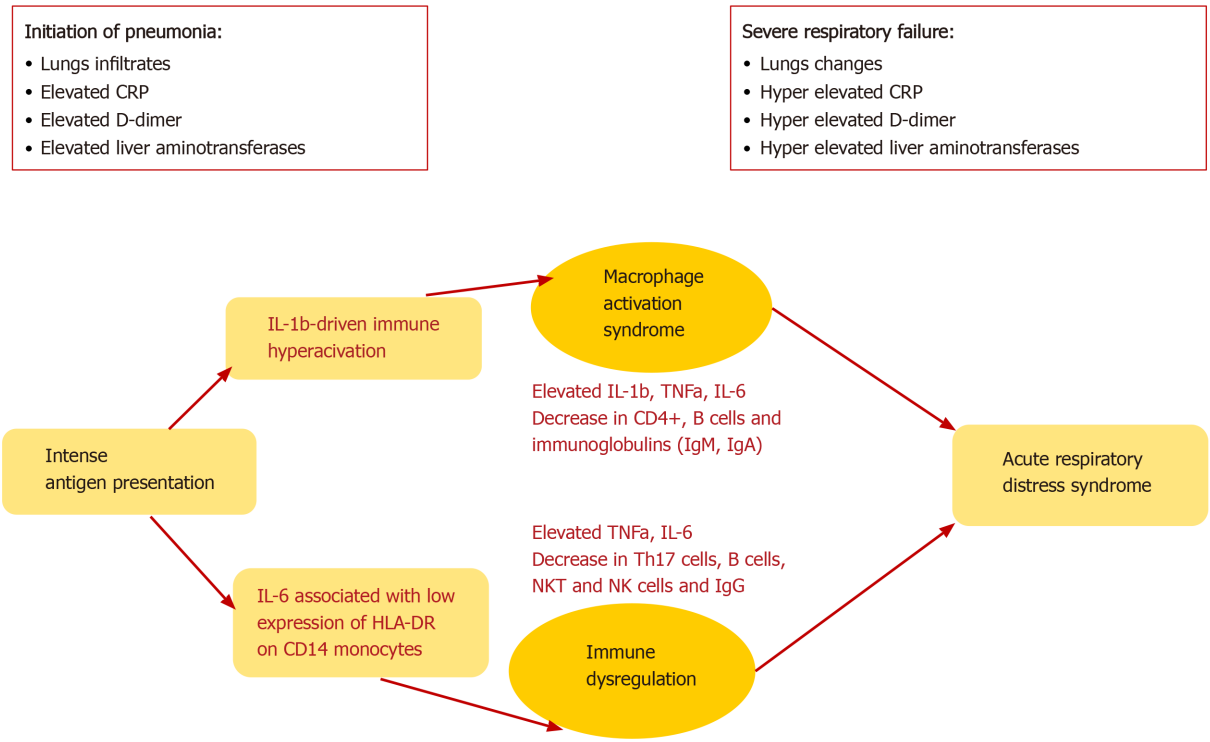

Figure 2 The immune dysregulation and macrophage activation syndrome, caused by IL-1b- and IL-6-driven hyperactivation, accompanied by a decrease in many cell types, both leading to acute respiratory distress syndrome. CRP: C-reactive protein; HLA: Human leukocyte antigen; Ig: Immunoglobulin; IL: Interleukin; Th: T helper; TNFα: Tumor necrosis factor alpha.

death in severe cases of coronaviral infections[58].

Another hypothesis on the severity of COVID-19 disease includes the problem associated with the activation of bradykinin B1 receptors on the lung endothelial cells. It is assumed that the enzymatic activity of the ACE-2 receptor inactivates des-Arg9 bradykinin, which is a ligand for B1. Furthermore, unlike B2, B1 is regulated by pro-inflammatory cytokines[59]. Interestingly, without the inactivation of B1 ligands, increased local vascular permeability is observed in lung mucosa, leading to angioedema. Probably, angioedema is a typical manifestation of the severe disease onset and the cause of typical changes visible on the computed tomography scan. The feeling of choking is observed mainly around day 9, denoted the worsening of the patients[60]. As any common viral infection, here, we also observed a progressive inflammatory condition with elevated IL-6, CRP, and ferritin but not procalcitonin or erythrocyte sedimentation rate, which indicates the second stage of the disease[60]. In line with this, the renin-angiotensin system could also contribute to the lung injuries along with sepsis-associated DIC. However, the levels of platelets, prothrombin time, and fibrinogen may remain normal[61].

The pathophysiology of the disease may be a combination of injury of pulmonary type II pneumocyte, viral pneumonia, ARDS, DIC, sepsis, cytokine storm, MAS, and overall immune dysregulation[62,63]. Some of these aspects are shown in Figure 2.

IMMUNE MEMORY AND VACCINE DEVELOPMENT AGAINST SARS-COV-2: DESIRE OR REALITY?

One of the main immunological features of COVID-19 is the exhaustion of lymphocytes. This, along with the reduced functional diversity of immune cells, is associated with a severe course of the disease[64]. The impaired immune response makes the possibility of adequate immune memory questionable. However, recovered patients displayed some SARS-CoV-specific antibodies up to 2-6 years after infection, unless they experienced ARDS, which led to undetectable peripheral memory B cell

responses[40,65]. It is not yet known whether the T cell response is enough for the protection of reinfection[66]. To develop its own specific protective anti-viral immune response during the incubation period and in mild cases, the host must be in good general health and have an appropriate genetic terrain (*e.g.*, certain HLA antigens). Moreover, genetic variants are known to contribute to individual differences in the immune response to pathogens.

But this is especially important when designing a vaccination strategy because an effective and protective vaccine depends desperately on the possibility of having immune memory after virus encountering[66].

Usually, COVID-19 survivors developed antibodies from 2-15 d after developing symptoms, as the immune response is broadly reminiscent of the typical anti-viral reaction[67]. The majority of patients produced antibodies against the spike protein[68]. Moreover, it was shown that the levels of antibodies correlated with patients age and the severity of the disease. The drawback of testing antibodies is that in mild cases, it is more likely not to produce detectable antibodies. Besides, it is not known whether these antibodies are protective or not. Some *in vitro* experiments with anti-SARS-CoV-2 antibodies showed that they are neutralizing and prevent the virus from entering the host cells[7]. However, we always have in mind that cellular immunity against the virus is more critical for viral clearance than the humoral. Thus, the role of Th, T cytotoxic, and NK cells should not be neglected. Studies that included survivors from the 2003 SARS epidemic have recently shown that neutralizing antibodies were found, 17 years after the infection[69]. Similar results were reported for the MERS epidemic, although the levels of neutralizing antibodies faded significantly after 5 years.

Furthermore, it was shown that people exposed for the second time to the virus developed much milder symptoms, even though reinfection can occur[70]. The reason for the decline in the antibodies levels remains unclear, even though some hypotheses are related to the short-life of memory B cells, particularly to coronavirus, hiding in the secondary lymphoid organs, such as lymph nodes and the spleen as well as in the bone marrow and the lungs. Interestingly, except for the antibodies levels, memory B cells can also be isolated and investigated as a marker of previous encountering the virus. Furthermore, some of the memory B cells of some patients reacted to the proteins from the live virus *in vitro*[71].

Here, we come to the myth of immune passports for COVID-19. The decision to make immune passports for people traveling from one country to another for tourism and recreation is not meaningless, but it exists only in theory because the real situation during the COVID-19 pandemic has been identified as more complicated where the "immune passport" cannot be a solution.

Let us look at the facts; if you go somewhere outside your country and carry the immune passport, you would guarantee that you are healthy and have antibodies to SARS-CoV-2. Especially in pandemics, the security of a society is the number one goal, where every breakthrough exposed the society to severe consequences. False-positive and negative results give some discrepancies and difficulties in interpretations. Moreover, the probability of false peace of mind that "I'm not infected, I can fly/travel safely" and not to keep the protective measures, immune passports are more valuable not as a medical document but as a business card. In such a way, the immune passports not only resolve the problem but create another, the more rapid spread of the infection.

It is scientifically challenging to claim that any given person will be protected from the infection if they reach a specific level of antibodies on testing. Any predictions based on the antibody presence carry legal and ethical issues. This false feeling of protection could also encourage dangerous behavior, such as refusal to wear masks and keep social distancing. Moreover, there will be people who would intentionally try to get sick to re-enter normal life.

Another aspect of the unclear immune response and immune memory regarding SARS-CoV-2 infection is the desire of the world to have an effective vaccine against the virus to be invented. This vaccine is essential to reduce the severity of the disease and its spread and clear the virus, thus helping to control current and future coronavirus outbreaks. There are several strategies for developing vaccines against SARS-CoV and MERS-CoV tested in animals. Among them are: Using live attenuated virus, viral vectors, inactivated virus, vaccine subunits, recombinant DNA, and protein vaccines[71]. There may currently be other promising targets to use in the creation of vaccines against SARS-CoV-2 infection and therapy, but additional laboratory and clinical evidence are still needed. Some new pharmaceutical drugs, including anti-human immunodeficiency virus and stem cell drugs, have been shown in these clinical trials. These studies are ongoing, but still it will take months to years to develop vaccines for SARS-CoV-2.

In summary, it is worrying that after discharge from the hospital, some patients remain positive for the virus, while others return with a relapse. This suggests that the immune response to SARS-CoV-2 intended to neutralize and eliminate the virus may be insufficient in at least some of the patients. Furthermore, this observation can predict that vaccines may not be effective in these individuals. Recovered patients who have not reached a severe stage should be monitored for the presence of the virus along with the measurement of T/B cell responses. All of these scenarios need to be considered when defining vaccine development strategies. Besides, since there are many types or subtypes of coronavirus, vaccines aimed directly at SARS-CoV-2 seems to be challenging to develop. Therefore, Edward Jenner's approach should be considered. In other words, the development of a vaccine against the whole family of CoV or against those representatives that cause disease only in animals.

THERAPY OPTIONS RELATED TO THE IMMUNE MECHANISMS

COVID-19 infection revealed some treatment options related to the immune responses. If we accept the division of SARS-CoV-2 infection into three stages, at the beginning of the infection (stage I, an asymptomatic incubation period with or without detectable virus), some of the mechanisms of innate immunity play a role, including NK cells, interferon production, and some cytokines. Therefore, strategies to boost immunity, such as administration of anti-serums (ready-made antibodies from survivors) or pegylated IFNα, are undoubtedly crucial at this stage.

During the incubation period, as well as the non-severe stage (stage II, a mild symptomatic period with the presence of a virus in the body), a specific adaptive immune response is required to neutralize and eliminate the virus, which will eventually prevent disease progression to severe stages. The adaptive response, however, occurs more slowly and is activated at a later stage. When the protective immune response is compromised, the virus will spread, and massive damage of the affected tissues and organs will occur, especially in those that highly express ACE-2 receptors, such as the gut and kidneys. This leads to lung inflammation, mostly mediated by pro-inflammatory immune cells. Inflammation of the lungs is the leading cause of life-threatening respiratory failure in the severe stage of the disease (stage III, severe respiratory symptomatic stage with high viral load). Therefore, once severe lung damage occurs, attention should be moved to the inflammation and the efforts to suppress the immune responses and control the symptoms[72]. Some of the treatment options related to the immunological mechanisms could be seen in Figure 3.

Antibody and plasma therapy are the next therapy option, closely related to the immune mechanisms. It has been reported that many cured patients donate plasma against SARS-CoV-2, as there were clinical trials for SARS-CoV[73] and MERS-CoV[74]. Preliminary data indicate favorable results for use in patients with acute and severe SARS-CoV-2 infection. In addition, the development of a recombinant human monoclonal antibody, such as CR3022, is a reasonably easy way to neutralize the virus by attachment to the receptor-binding domain of SARS-CoV-2. This SARS-specific human monoclonal antibody has the potential to be developed as a drug for SARS-CoV-2 infection[75]. Other monoclonal antibodies that neutralize SARS-CoV, such as m396 and CR3014, may be alternatives for the treatment of SARS-CoV-2[76].

Passive antibody therapy is the administration of ready-made antibodies against an infectious agent to a susceptible individual to prevent or treat an infectious disease caused by that microorganism. Thus, the passive application of antibodies is the only means of ensuring the immediate and ready immunity of endangered persons. Experience with previous epidemics with other CoV, such as SARS-CoV-1, has shown that survivors' sera may contain neutralizing antibodies to the virus, thus the expected mechanism of action of passive antibody therapy is viral neutralization and elimination. However, other mechanisms, such as antibody-dependent cellular cytotoxicity and/or phagocytosis, are also possible[72].

Currently, the only ready-to-use antibodies are those obtained from survived patients. As more people become ill with COVID-19 and recover, the number of potential donors will continue to increase. The serum of recovered individuals can be used prophylactically to prevent infection in high-risk individuals, such as those with chronic diseases, medical personnel, and those who have been in contact with patients. The efficacy of this approach is unknown, but historical experience has shown that products containing passive antibodies are more effective in preventing than in treating existing disease.

If used for therapy, antibodies are most effective when administered soon after the

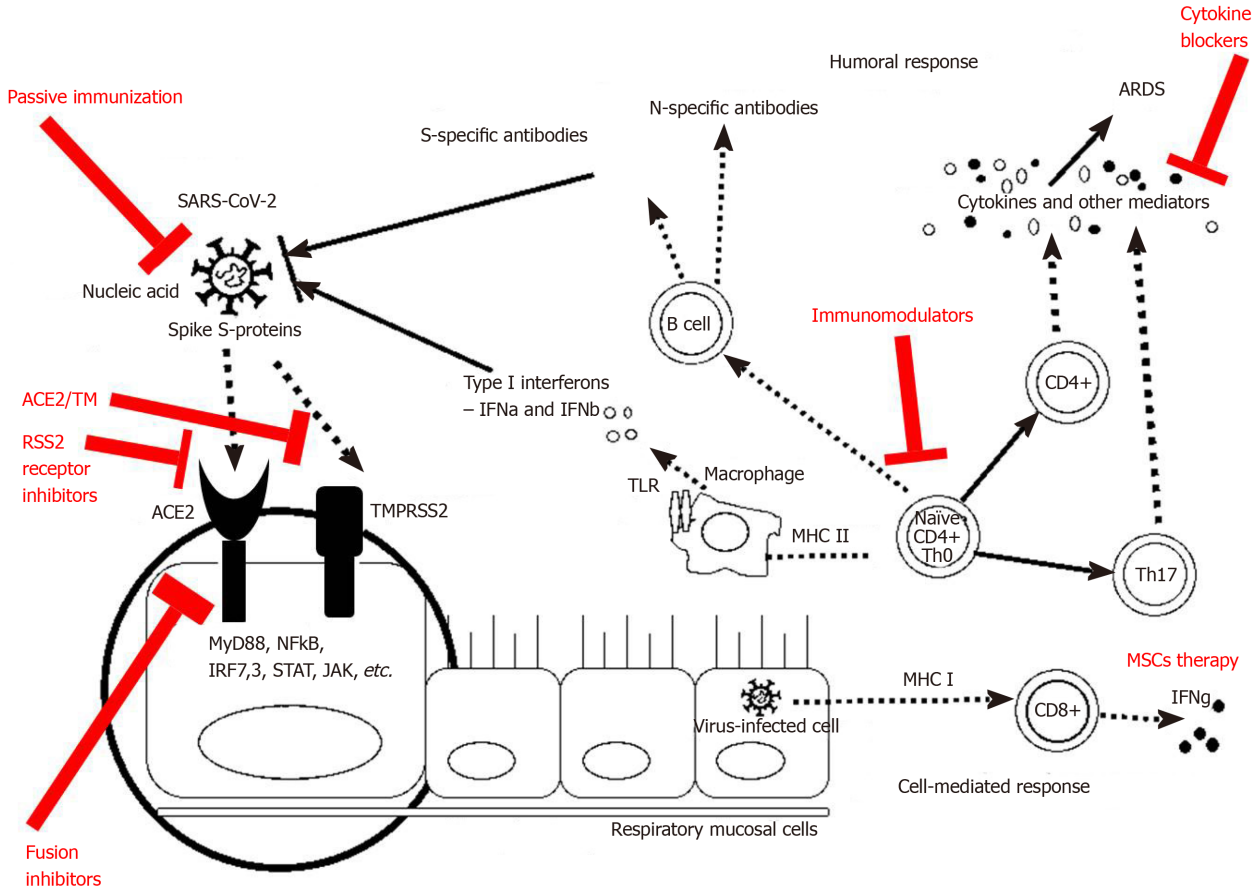

Figure 3 Treatment modalities related to the immunological mechanisms observed during coronavirus disease 2019. ACE2: Angiotensin-converting enzyme 2; ARDS: Acute respiratory distress syndrome; MHC: Major histocompatibility complex; MSCs: Mesenchymal stromal/stem cells; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2.

onset of symptoms. The antibody acts by altering the inflammatory responses, which is also more easily achieved during the initial immune response, or the asymptomatic stage. The reason for the differences in efficacy is not well understood. Still, it may reflect the fact that the antibody works by neutralizing the initial inoculum from the infectious agent, which is probably much less than that in an already developed infection and a large number of viral copy. In line with this, to be effective, a sufficient amount of antibodies must be administered. When given to a person at risk of infection, this antibody will reach the tissues *via* blood and can provide protection against the infection. Depending on the amount and composition of immunoglobulins, passive protection can last from weeks to months (for IgG). The challenges, however, are related to the difficulties in that, as we have mentioned above, some patients do not possess high antibody titers after the illness. Therefore, due to the individual variation of the immune responses, the insufficiently active immune response in some people will be a reason for them to be prone to reinfection.

In a prophylactic regimen of the use of passive immunotherapy in persons at risk, the aim is to prevent disease. Used therapeutically, the passive serum is administered to patients with clinical manifestations of the disease to reduce the severity of symptoms and mortality. The efficacy of these approaches cannot be measured without conducting a controlled clinical trial[72].

The risks of passive application of sera products fall into two categories - known and theoretical. The known risks are those associated with the use of blood products - infectious diseases and allergic reactions to the components of the serum, including serum sickness. With modern blood banking techniques, these risks are low. For sera used for therapy, there are also theoretical risks for transfusion-related acute lung injury and antibody-dependent enhancement (ADE) of infection. Several mechanisms of ADE have been described for CoV, and there are concerns that antibodies against one type of coronavirus may exacerbate infection to another virus strain. It is possible to experimentally predict the risk of ADE in SARS-CoV-2, as suggested for MERS. It is

assumed that when using sera rich in virus-neutralizing SARS-CoV-2 antibodies, the risk of developing ADE is minimal[72]. However, it is good to test these hypotheses in clinical trials. Another theoretical risk is that the use of antibodies to individuals exposed to SARS-CoV-2 may weaken the immune response so that not enough of their own antibodies would form. This puts these people at risk for subsequent reinfection. However, if the risk proves to be real, these individuals can be vaccinated against COVID-19 when the vaccine becomes available.

The preparation of highly purified and enriched neutralizing antibodies against SARS2-CoV-2 is preferable because they are safer and have higher activity. However, such preparations will not be available in the coming months, while locally derived serums may be available much sooner[77].

Other therapy strategies are oriented against the cytokine storm. Because lymphocytopenia is commonly seen in severe cases of COVID-19, the cytokine storm caused by the SARS-CoV-2 virus may be mediated by leukocytes other than T cells. High white blood cell counts are common, which, along with lymphocytopenia, is used as a differential diagnostic criterion for COVID-19. In any case, the blocking of IL-6 can be effective, as well as the blocking of IL-1 and TNFα. The approach to blocking these pro-inflammatory cytokines has been adopted in various autoimmune diseases. We also proposed anti-IL-6 therapy in IBD patients as a way of limiting the inflammation and development of colorectal carcinoma[78].

Anti-inflammatory strategies, including the blockade of specific cytokines that increase B1 expression on endothelial cells locally at the site of inflammation in combination with B1 and or B2 receptor blockade, should be considered. IL-1 (consisting of IL-1α and IL-1β) and TNF are potent inducers of the B1 receptor. Blocking the translocation of nuclear factor kappa B, TNF-α, or IL-1 prevents the regulation of B1 receptors both functional and molecular by lipopolysaccharide. Therefore, one strategy could be the treatment with anakinra, a monoclonal antibody that blocks not only IL-1α but also IL-1β, and has an excellent safety profile. IL-1a is probably in extremely high concentrations locally due to its release from damaged cells. TNF blockade is an option but is associated with many more infectious complications. Also, complement activity has been described and may play a role in this stage of the disease. Moreover, it may be affected by blockade of C5 component with eculizumab, a monoclonal antibody that was randomized to COVID-19 (NCT04288713)[60]. The use of antibodies against cytokines is a general approach in several autoimmune and autoinflammatory diseases in which one or more cytokines play a direct role in the pathogenesis. However, this approach is not without any side effects, so the benefit should always be considered.

On the other hand, a significant drawback is the fact that blocking of a cytokine rarely has the desired effect because the cytokines are connected in a network. Corticosteroids are also an option for therapy in these cases. Anti-inflammatory strategies may provide time, but will not cure the disease on their own if the virus is present or bradykinin-associated angioedema is not controlled[60].

Although various clinics in China have proclaimed the use of mesenchymal stromal/stem cells (MSCs) in severe cases of COVID-19 infection, no reliable results have yet been published. One of the advantages of this therapy is that MSCs must be activated by IFNγ. After that, MSCs are capable of exerting their anti-inflammatory effects. By producing various growth factors, MSCs can help repair damaged lung tissue. However, severely affected patients may lack these MSCs properties because T cells do not activate well in SARS-CoV-2 infection. The use of a "licensing approach" is being considered: Pre-treatment of MSCs with IFNγ with or without TNFα or IL-1. Thus, cytokine-treated MSCs may be more effective in suppressing the hyperactive immune response and promoting tissue repair, as such MSCs are useful in acute lung damage caused by lipopolysaccharide[72]. In any case, the use of MSCs in clinical practice should be limited to strict indications, taking into account the benefit-risk for each patient. Their oncogenic potential should not be neglected.

CONCLUSION

Based on the COVID-19 results from the interaction between the SARS-CoV-2 virus and the individual's immune system, we can assume that the onset and development of COVID-19 significantly depend on this communication.

Viral factors, such as viral type, load, titer, *in vitro* viability, mutations, as well as individuals' factors like genetics, age, gender, overall health, nutritional status, neuroendocrine-immune regulation, *etc.*, can determine the duration and severity of

the disease, probably the risk of reinfection.

Now, the science is directed to acquiring new data on the immunology, including the immune memory against the virus, the development of new technologies for the detection of infection, and effective vaccines. However, the unknown is much greater than verified knowledge of the SARS-CoV-2 virus and COVID-19.

Being a global health issue, COVID-19 urges scientists to put a lot of effort into developing effective therapeutic strategies for treating and saving lives. Little is known about the histopathological changes in the lungs and the damaged organs, as there are few reports of postmortem examinations. Thus, further research would be of great significance for the detailed understanding of SARS-CoV-2 infection.

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REVIEW

Targeting the phosphoinositide-3-kinase/protein kinase B pathway in airway innate immunity

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Abstract

The airway innate immune system maintains the first line of defense against respiratory infections. The airway epithelium and associated immune cells protect the respiratory system from inhaled foreign organisms. These cells sense pathogens *via* activation of receptors like toll-like receptors and taste family 2 receptors (T2Rs) and respond by producing antimicrobials, inflammatory cytokines, and chemokines. Coordinated regulation of fluid secretion and ciliary beating facilitates clearance of pathogens *via* mucociliary transport. Airway cells also secrete antimicrobial peptides and radicals to directly kill microorganisms and inactivate viruses. The phosphoinositide-3-kinase/protein kinase B (Akt) kinase pathway regulates multiple cellular targets that modulate cell survival and proliferation. Akt also regulates proteins involved in innate immune pathways. Akt phosphorylates endothelial nitric oxide synthase (eNOS) enzymes expressed in airway epithelial cells. Activation of eNOS can have anti-inflammatory, anti-bacterial, and anti-viral roles. Moreover, Akt can increase the activity of the transcription factor nuclear factor erythroid 2 related factor-2 that protects cells from oxidative stress and may limit inflammation. In this review, we summarize the recent findings of non-cancerous functions of Akt signaling in airway innate host defense mechanisms, including an overview of several known downstream targets of Akt involved in innate immunity.

Key Words: Lung; Nitric oxide synthase; Nuclear factor erythroid 2 related factor-2; Respiratory infections; Cystic fibrosis

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Core Tip: The human respiratory epithelium is continuously exposed to pathogens during each inhalation. Protection of the lung depends on complex signaling networks that activate host defense mechanisms. The kinase protein kinase B (Akt) interacts with numerous cellular proteins involved in airway innate immunity. In this review, we discuss the Akt pathway and known downstream targets involved in airway innate immunity.

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INTRODUCTION

The respiratory epithelium is the first-point of defense in the respiratory system, which is continuously exposed to a wide variety of inhaled pathogens. The classic role of the respiratory epithelium in airway defense is clearance of inhaled microbes *via* ciliary beating and mucociliary clearance (MCC)[1]. However, the respiratory epithelium is complex and contains not only epithelial cells but also resident macrophages, dendritic cells, and other leukocytes. All of these cells sense infection through protein receptors like toll-like receptors (TLRs) and taste family 2 receptors (T2R) bitter taste receptors[2] which activate production of a wide variety of antimicrobial peptides and radicals as well as inflammatory cytokines and chemokines. For example, stimulation of TLRs on airway epithelial cells regulates the expression of genes encoding multiple cytokines and antimicrobial peptides[3] *via* nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) signaling[4]. Activation of the innate immune system thus stimulates adaptive immunity and is also associated with apoptosis and other signal transduction pathways.

One understudied protein in airway innate immunity is protein kinase B, also known as Akt, a widely expressed serine/threonine kinase[5]. Activation of Akt by upstream kinases such as phosphoinositide-3-kinase (PI3K) stimulates phosphorylation of downstream targets involved in cell proliferation, apoptosis, and/or cell growth depending on the signaling context. Akt has also recently been suggested to play a role in innate immunity by regulating immune cell development, survival, and function[6].

Akt also has other targets important for innate immune responses of the airway epithelial cells themselves. Akt phosphorylates and activates endothelial (e) nitric oxide synthase (NOS), an enzyme that produces nitric oxide (NO)[7]. NO has many biological functions in the airway, including activating smooth muscle relaxation, increasing ciliary beat frequency, and having direct bactericidal and anti-viral effects[8]. Another target activated by Akt is nuclear factor erythroid 2 related factor-2 (Nrf-2), a transcriptional factor that drives the expression of antioxidant genes that can protect against oxidative stress-induced by microbes as well as over-active inflammatory pathways[9].

Abnormalities of innate immunity are linked with numerous airway diseases including chronic rhinosinusitis[10-12] and cystic fibrosis (CF)[13]. This review focuses on the Akt-dependent regulation of innate immunity in the lung and the potential role of Akt in protecting the airways against infection. Emphasis will be placed on novel directions for drug development. Furthermore, we summarize the current understanding of the role of the airway epithelium in Akt-dependent innate immunity and host defenses against bacterial infections in CF.

OVERVIEW OF THE PHOSPHOINOSITIDE-3-KINASE / PROTEIN KINASE B PATHWAY

Akt was identified almost 30 years ago by its homology to the v-akt retroviral oncogene[5] and subsequently found to be a 57 kDa serine and threonine protein kinase that plays a pivotal role in cell proliferation, survival, and death[14,15] (Figure 1). There are three conserved mammalian Akt isoforms: Akt1 (PKBa), Akt2 (PKBb), and Akt3 (PKBg). Some Akt isoform knockout studies conducted in mice have suggested there

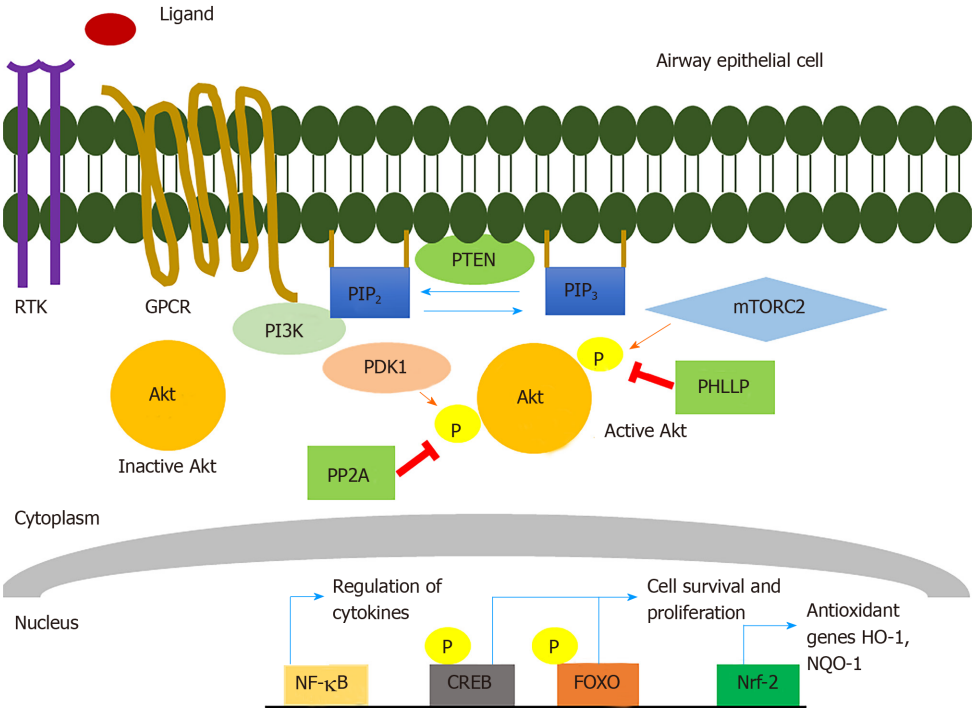

Figure 1 Protein kinase B signaling pathway. Stimulation of receptor tyrosine kinases or G-protein-coupled-receptors activates phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) which phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP_2) at the plasma membrane to generate PIP_3. Inactive Akt in the cytosol gets recruited to the plasma membrane where it gets phosphorylated at T308 in the kinase domain by phosphoinositide dependent kinase 1 and at S473 in the regulatory domain by mTORC2 resulting in full activation; Signal termination is achieved by PTEN where it dephosphorylates PIP_3 to PIP_2; Additionally, PP2A and PHLPP have shown to regulate Akt kinase activity by direct dephosphorylation; Activation of Akt is known to regulate crucial transcription factors such as nuclear factor-kB (NF-kB), CREB, FOXO, and Nrf-2, each of which regulates a variety of target genes that regulate cell survival, proliferation, differentiation, migration, and metabolism; Akt is known to phosphorylate IkB kinase which phosphorylates IkB-a releasing NF-kB to translocate into the nucleus and transcribe genes; Activation of Akt might increase or suppress NF-kB regulated genes (IL-8, IL-18) depending on the stimulus. Both FOXO and CREB are known to regulate apoptosis and phosphorylation of these transcription factors by Akt, has shown to control cell survival. Nrf-2 activation by Akt increases the production of antioxidant genes such as HO-1 and NQO-1 that counteracts oxidative stress and inflammation. RTK: Receptor tyrosine kinase; GPCR: G-protein-coupled receptor; PI3K: Phosphoinositide-3-kinase; PIP_2: Phosphatidylinositol 4,5-bisphosphate; Akt: Protein kinase B; PDK1: Phosphoinositide dependent kinase 1; mTORC2: Mammalian target of rapamycin; PTEN: Phosphatase and tensin homolog; PHLPP: PH domain and leucine rich repeat protein phosphatases; NF-kB: Nuclear factor kappa-light-chain-enhancer of activated B cells; CREB: cAMP response element binding protein; FOXO: Forkhead family of transcription factors; Nrf-2: Nuclear factor erythroid 2 related factor-2; PP2A: Protein phosphatase 2; HO-1: Heme oxygenase; NQO-1: NADPH quinone dehydrogenase 1.

may be specific functions for certain isoforms in growth, metabolism, and development, though this may be in large part due to differences in the tissue distributions of the isoforms[15,16]. All three isoforms of Akt contain an N-terminal Pleckstrin Homology (PH) domain, a kinase domain, and a C-terminal regulatory domain. Activation of some receptor tyrosine kinases (RTKs) and/or some G-protein-coupled-receptors (GPCRs) by growth factors such as insulin-like growth factor-1 can activate the Akt pathway *via* plasma membrane recruitment and activation of class I PI3K isoforms[15,17]. Activated PI3K can phosphorylate phosphatidylinositol 4,5-bisphosphate (PI4, $5P_2$) to generate phosphatidylinositol (3,4,5)-trisphosphate (PIP_3). Inactive cytosolic Akt gets subsequently recruited to the membrane *via* the interaction of PIP_3 with the Akt PH domain. Akt can also be recruited to the membrane by PI3, $4P_2$ produced by class II PI3K phosphorylation of PI4P[15].

Akt localization to the plasma membrane induces conformational changes that allow phosphoinositide-dependent protein kinase-1 (PDK-1) to phosphorylate threonine (T) 308 within the activation loop of the Akt1 kinase domain (corresponding to T309 and T305 in Akt2 and Akt3, respectively) and mTOR Complex 2 (mTORC-2) to phosphorylate serine (S) 473 within the hydrophobic C-terminal Akt regulatory domain (corresponding to S474 and S471 in Akt2 and Akt3, respectively)[15]. Maximal activation of the kinase requires phosphorylation of both residues[15]. Multiple other phosphorylation sites exist in Akt that can be phosphorylated by kinase complexes like mammalian target of rapamycin (mTORC2) (T450 in Akt1), CK2 (S129 in Akt1) GSK-3a (T312 in Akt1), cyclin A-CDK2 (S477 and T479 in Akt1), though how they modulate Akt activity is less clear[15].

Once Akt is activated, it can phosphorylate multiple downstream targets and/or redistribute to many cellular compartments, including the nucleus[18]. A study done

with a genetically-encoded fluorescent biosensor for Akt activity showed that its activity in the nucleus was less rapid but more sustained compared with cytosolic Akt activity[19], suggesting that Akt can be regulated differently in the cytosol vs nucleus or other organelles. Activation of Akt is also negatively regulated by phosphatases, including phosphatase and tensin homolog (PTEN) that antagonizes PI3K signaling by dephosphorylating PIP_3 and converting it back to PI4, $5P_2$. Protein phosphatase 2 (PP2A) and PH domain and Leucine-rich repeat Protein Phosphatases (PHLLP) also reduce Akt activation by dephosphorylation at T308 and S473, respectively[15].

Dysregulation of the PI3K/Akt pathway is associated with diabetes, cancer neurological disorders, and cardiovascular diseases[15]. Numerous studies have reported that components of the Akt signaling pathway are frequently mutated in multiple types of cancer; in some cases, this is associated with tumor aggressiveness[18]. In many tumors, Akt activity is upregulated *via* one or more mechanisms including loss of PTEN, mutations in the PI3K catalytic subunit, or loss of expression of phosphatases such as leucine rich repeat protein phosphatases (PHLPP)1 and PHLPP2 that dephosphorylate Akt[15,20].

Beyond the well-known functions of Akt in cell proliferation and survival and, consequently, the pathophysiology of cancer, the Akt pathway has several roles in the immune system. Akt signaling is important for the maturation and survival of dedicated immune cells. Activation of the Akt pathway is a necessity for the development of human dendritic cells (DCs)[21] and survival of activated B cells[22]. Furthermore, within airway epithelial cells, there are many known downstream targets of Akt that have important innate immune functions. The below sections will describe the innate immune system of the respiratory epithelium and importance of some known Akt targets in airway innate immunity.

OVERVIEW OF RESPIRATORY INNATE IMMUNITY

The innate immune system is the first line of defense against potentially dangerous microbes, and its main role is to recognize pathogens and initiate fast defensive responses. Because the human respiratory tracts are exposed to a myriad of pathogens daily, the immune system needs to recognize and initiate host defenses against these pathogens[11]. Akt may regulate multiple points of the airway innate immune system as well as the airway's ability to detect pathogens.

Mucociliary clearance: The physical defense of the airways

The primary physical innate defense mechanism of the airways is mucociliary clearance (MCC) (Figure 2). The main functional components of MCC are mucus production by airway secretory cells[23] and mucus transport by airway ciliated cells[11,24-26]. Cilia are specialized organelles lining airway epithelial cells. Mucus traps inhaled particulates and pathogens, and coordinated ciliary beating drives debris-laden mucus toward the pharynx, where it is swallowed or expectorated[27]. The airway surface liquid (ASL) is composed of the mucus layer that rides on top of the periciliary liquid (PCL) that surrounds the cilia. The composition of the PCL (volume, viscosity, and pH) mainly depends on epithelial ion channels[28]. Dysregulation of epithelial ion channels in CF is associated with increased mucus viscosity and PCL depletion[29] that impairs MCC. Direct cilia motor protein defects in primary ciliary dyskinesia (PCD) also impair MCC. Both CF and PCD patients are more susceptible to airway infections[30-32], supporting the importance of effective MCC to airway defense. A reduction of ciliated cells is also observed in patients with inflammatory diseases like chronic rhinosinusitis[32,33] as well as after exposure to compounds in cigarette smoke[24].

The normal mucus layer is composed of mainly water, mucins, proteins, lipids, and salts. However, the gel properties of mucus are produced by mucins, large cross-linked glycoproteins, including mucin 5AC (MUC5AC) produced by surface goblet cells[34] and MUC5B produced by mucus cells of submucosal glands[35]. Elevated MUC5AC levels are linked to asthma and may contribute to airway obstruction[36-38]. Akt has been suggested to be linked to MUC5AC production, though the data are conflicting. In human bronchial epithelial cells, direct inhibition of Akt upregulates MUC5AC production[39]. Activation of the PI3K/Akt pathway may also significantly reduced influenza-induced MUC5AC overproduction *via* negative cross-talk with the mitogen-activated protein kinase (MAPK) pathway[40]. In contrast, other studies showed that inhibition of Akt reduces MUC5AC levels[41,42]. The discrepancies in these studies might be due to different experimental models used. However, because Akt

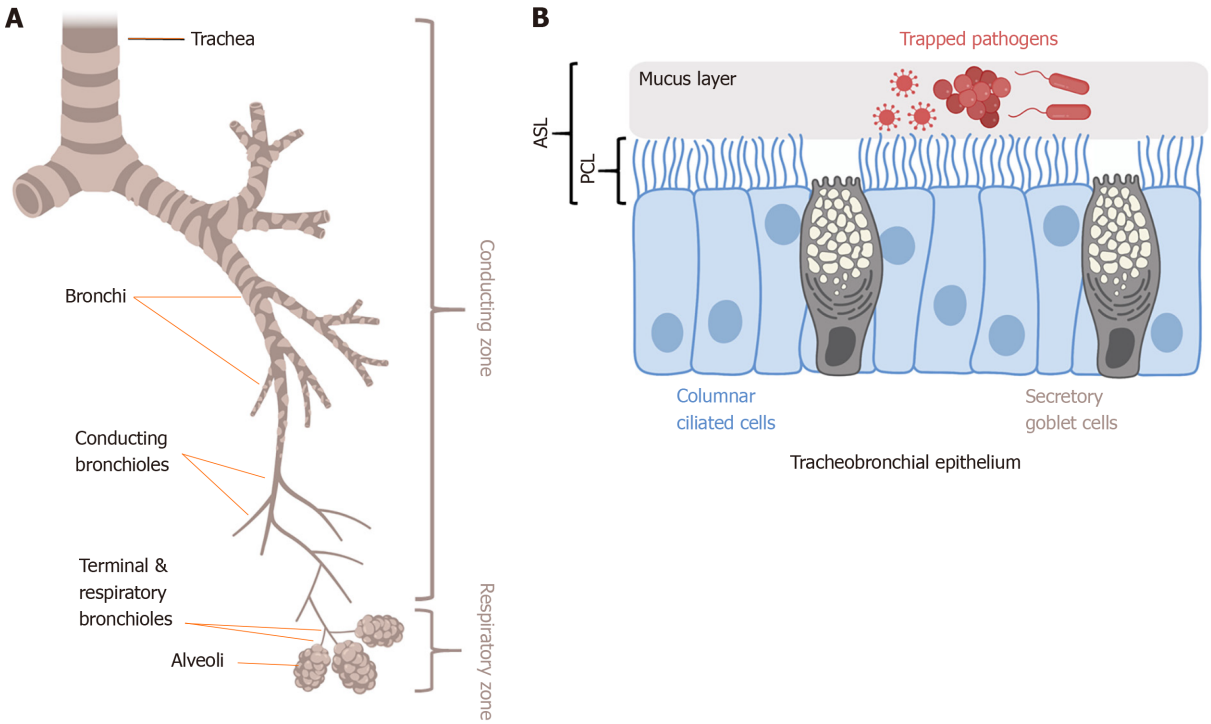

Figure 2 Mucociliary clearance and innate immunity in the lung. A: Trachea, bronchi, and conducting bronchioles comprise the conducting zone of the airways; B: The conducting airway epithelium is lined with columnar motile ciliated cells and secretory goblet cells. Goblet cells secrete mucins like MUC5AC that polymerize to form mucus, which traps inhaled pathogens and debris; The mucus layer rides on top of a less viscous PCL composed of salt, water, and antimicrobials; Together, the mucus and PCL comprise the airway surface liquid; Coordinated metachronal beating of the motile cilia within the PCL layer pushes the sticky mucus layer up to respiratory tree to the oropharynx, where it is expectorated or swallowed; This process is termed MCC, and is the physical defense of the airway against infection; Epithelial cells also secrete antimicrobial peptide and radicals (NO, H_2O_2) to directly kill pathogens and produce cytokines and chemokines to activate inflammation; Shown is a representative diagram of tracheal or bronchial epithelium; In lower conducting airways (non-cartilaginous bronchioles < approximately 1 mm in diameter), secretory club cells (also known as bronchiolar exocrine cells) are found instead of goblet cells; As described in the text, there are several potential mechanisms by which protein kinase B may regulate MCC and other innate immune pathways. Figure made using Biorender.com. PCL: Periciliary layer; MCC: Mucociliary clearance; MUC5AC: Mucin 5AC.

may play a role in regulating MCC by controlling MUC5AC levels, Akt inhibitors or activators may be a novel therapeutic strategy to manipulate MUC5AC levels to reduce mucus hypersecretion in asthma or chronic obstructive pulmonary disease (COPD).

Immune surveillance receptors in the airway

Beyond the airway's physical defenses, Akt is also involved in immune surveillance in the airway. The airway utilizes a gamut of receptors such as toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), and T2R bitter receptors to detect invading pathogens[10,43-45]. TLRs are pattern recognition receptors (PRRs) initially discovered based on homology to *Drosophila* toll receptors[46]. TLRs recognize pathogen-associated microbial patterns (PAMPs) and activate signaling pathways that can lead to increased transcription of cytokines as well as production of antimicrobial peptides and iNOS[47]. Dysfunction of TLR signaling has been linked to COPD, acute lung injury, CF, and CRS[10,48-50].

In humans, 11 TLRs have been identified and are involved in the innate sensing of microbial products[51]. These TLRs are found in dedicated immune cells such as macrophages and dendritic cells. TLRs are also found in fibroblasts, epithelial cells in the lung, intestine, and many other cell types[10,11,52,53]. Primary and immortalized airway cells express TLRs 2 through 10 at varying expression levels[50,54-57]. Lung epithelial cell TLRs respond to a variety of factors such as *Pseudomonas aeruginosa* flagellin (*via* TLR5), gram-negative bacterial lipopolysaccharide (LPS; *via* TLR4), unmethylated CpG from prokaryotic DNA (*via* TLR9), bacterial peptidoglycan (*via* TLR2), gram-positive bacterial lipoteichoic acid (*via* TLR2), viral double-stranded RNA (*via* TLR3), and fungal zymosan/beta-glucan (*via* TLR2)[43,50,57].

The broad principles of TLR signaling are already described by several excellent reviews[58,59]. Briefly, binding of PAMPs to TLRs activate their intracellular Toll/IL-1 receptor (TIR) domains[2] and recruits one or more TIR domain-containing adaptor

proteins, including myeloid differentiation primary response protein 88 (MyD88), TIR-domain-containing adaptor protein (TIRAP), TIR-domain-containing adaptor protein inducing interferon-β (TRIF), and TRIF-related adaptor molecule (TRAM)[59]. Signaling then proceeds through a serious of adapter proteins. Association of MyD88 recruits IL-1R-associated kinase (IRAK)[60] through interactions of N-terminal death domains in both proteins[61]. Phosphorylation of IRAK activates tumor necrosis factor receptor-associated factor-6 (TRAF6) which in turn activates transcription factors such as NF-kB and JNK to promote the production of cytokines or initiate apoptosis signaling pathways, respectively[62]. Some TLRs, like TLR3, can also activate MyD88-independent signaling pathways leading to NF-kB activation[63].

In epithelial and immune cells, experimental studies have identified both positive and negative cross-talk between TLR activation and the PI3K/Akt pathway[64-66]. It is not yet fully understood how Akt is linked to TLR signaling, and these links maybe cell type-dependent or even TLR-isoform-dependent. PI3K, upstream of Akt, is often activated by TLRs in many cells[67], with Akt phosphorylation peaking at approximately 20 min and decreasing by approximately 1 h after stimulation[68]. Activation of Akt *via* TLR stimulation may increase NF-kB signaling and cytokine expression in macrophages[65], while other studies showed that the PI3K/Akt pathway suppresses TLR-induced cytokine secretion in monocytes *via* inhibition of NF-kB[69-71]. One study suggested that binding of vasoactive intestinal peptide (VIP) to GPCRs reduced TLR4 expression *via* Akt in macrophages and regulatory T cells[52,72,73]. Another group demonstrated the activation of PI3K/Akt after stimulation of TLR4 is crucial for B cell survival[22]. The role of Akt in airway TLR signaling is relatively unexplored, but data suggest that pharmacological manipulation of PI3K or Akt signaling may be a mechanism by which NF-kB activity could be controlled during bacterial or viral infection and the resulting activation of TLRs in the airway.

Cross-talk between TLRs and Akt maybe particularly important during cellular hyperoxia in the lung. Oxygen therapy is commonly used to reduce tissue hypoxia in patients with pulmonary disease. However, hyperoxia can induce lung damage that may be tied to a reduction of Akt signaling. Expression of a constitutively active form of Akt protected mouse lungs from hyperoxic injury[74]. In a rat model of bronchopulmonary dysplasia (BPD), exposure of neonatal lungs to high (95%) oxygen reduced the expression of Akt, while overexpression of Akt was protective against lung damage[75]. TLR4-deficient mice showed increased lung injury, higher mortality, and reduced levels of phospho (p)-Akt after hyperoxia. Expression of anti-apoptotic BCL-2 and activation of p-Akt significantly attenuated hyperoxia-induced lung injury in these TLR4-deficient mice[76]. Thus, activating the Akt pathway with receptor ligands or direct activators like SC-79[77] may be useful for treatment of lung injury during hyperoxia.

Other PRRs exist beyond TLRs. NLRs are PRRs that activate signaling pathways leading to activation of the inflammasome. Unlike the transmembrane TLRs, NLRs are cytosolic. NLRs can respond to microbial pathogens and stimulate the production of cytokines. Depending on the domains that are expressed, NLRs can be categorized as NOD receptors, NLRP, NLRC, or NLRB, and have been extensively reviewed[78,79]. NOD1 and NOD2 are expressed in lung epithelial cells, endothelial cells, alveolar macrophages, and airway smooth muscle cells[78]. Binding of NOD1 and NOD2 to secreted bacterial moieties results in activation of NF-kB, and polymorphisms of these receptors may increase susceptibility to respiratory infections[80]. NLRP3 may play a major role in recruiting neutrophils and dendritic cells during *Mycoplasma pneumoniae* lung infection in mice[81]. Because NLRs are relatively novel compared with TLRs, the knowledge of NLRs/Akt/PI3K/NF-kB in the lung immunity field is still rapidly developing.

Two decades ago, the GPCRs for bitter taste (known as taste family 2 receptors or T2Rs) were discovered in taste bud type II cells on the tongue[82]. There are 25 T2R isoforms in humans[82,83] that detect bitter compounds in food. However, in recent years, the discovery of the T2Rs in extraoral tissues has suggested other roles for these receptors beyond taste, including immune surveillance[83]. A variety of bitter receptors are expressed in the motile cilia in human airway epithelial cells[44] and macrophages[84] which are stimulated by bitter molecules such as denatonium benzoate[85], thujone from the wormwood plant[85], sodium thiocyanate[12], phenylthiocarbamide (PTC)[12], and bitter plant flavonoids[25]. These T2Rs also recognize gram-negative bacterial products such as acyl-homoserine lactone (AHL)[12] and quinolone[86] quorum-sensing molecules, suggesting they may play a role in sensing developing biofilms.

Stimulation of bitter receptors in sinonasal epithelial cell cilia activates Ca^{2+}-dependent nitric oxide (NO) production which is bactericidal[12]. Additionally, NO can act as a second messenger to stimulate soluble guanylyl cyclase (sGC) and protein

kinase G (PKG) to phosphorylate downstream effector proteins within the cilia and increase the ciliary beat frequency and thereby MCC[87]. One T2R isoform expressed in respiratory cilia is T2R38. Common polymorphisms in the *TAS2R38* gene that render the T2R38 receptor nonfunctional are associated with increased susceptibility to upper respiratory infection[12,88], susceptibility to chronic rhinosinusitis[89-94], and surgical outcomes after functional endoscopic sinus surgery[95].

T2Rs also play other roles in the airway. A different subset of T2R isoforms in non-ciliated solitary chemosensory cells (SCCs), sometimes called tuft cells[44,96], leads to the propagation of Ca^{2+} to neighboring ciliated cells *via* gap junctions, triggering the neighboring cells to release anti-microbial peptides such as beta-defensin 1 and 2[96,97], which can permeabilize fungi and both gram-positive and negative bacteria[44]. Moreover, in mouse asthma models, bitter receptor agonists are effective in reducing airway smooth muscle contraction by modulating Ca^{2+} signaling[98-100].

Such studies of primary cells *in vitro* and patients *in vivo* suggest that T2Rs may contribute to the recognition of bacterial products similarly to TLR signaling[45,86]. Since T2Rs activate endothelial nitric oxide synthase (eNOS) to acutely produce NO in ciliated cells, targeting this pathway through Akt, which phosphorylates and activates eNOS[101,102] independently of Ca^{2+}, as described below, is possibly a way to activate these innate immune responses in patients with polymorphisms that render specific T2Rs like T2R38 nonfunctional. Akt also has many other downstream targets, including Nrf-2[103] that play a role in the above innate immune processes. Several of these targets are reviewed below.

DOWNSTREAM TARGETS OF AKT INVOLVED IN INNATE IMMUNITY AND INFLAMMATION

Nitric oxide synthases

Nitric oxide synthase (NOS) enzymes catalyze the production of NO. L-arginine and NAD(P)H are converted to NO, NAD(P), and L-citrulline, requiring tetrahydrobiopterin (BH_4) as a co-factor. NO is an important physiological signaling molecule that regulates processes like pulmonary vascular tone. NO also stimulates sGC to produce cyclic GMP (cGMP), which then activates PKG[104]. PKG increases ciliary beat frequency to enhance MCC of detrimental pathogens, as described above[105]. Enhancing airway cell NO production by addition of L-arginine, adding artificial NO donors, introducing cell-permeant cGMP analogues, or using cGMP phosphodiesterase inhibitors to increase cGMP can all enhance ciliary beat frequency in rat, mouse, bovine, and human airway ciliated cells[60,106-111]; conversely, inhibition of NOS reduces the ciliary beat frequency in cultured ciliated airway epithelial cells[60].

There are three mammalian NOS isoforms; endothelial (eNOS, or NOS3), neuronal (nNOS or NOS1), and inducible (iNOS or NOS2) isoforms, named after the tissue where they were originally discovered. The main NOS isoform in neurons is nNOS[112], but nNOS has been detected in epithelial cells of various organs, pancreatic islets, and vascular smooth muscle, and exocrine acinar cells[113]. The dominant isoform in endothelial cells that maintains vascular tone and blood pressure is eNOS[101]. The airway epithelium generally expresses eNOS at baseline, while iNOS expression can be up-regulated during inflammation[11,25,101,106,110,114]. Pollutants and cigarette smoke can downregulate eNOS expression and subsequently reduce the production of NO[115].

Both eNOS (NOS3) and nNOS (NOS1) are generally constitutively expressed and are regulated acutely *via* binding of Ca^{2+}-bound calmodulin as well as phosphorylation, described below. Activation of airway epithelial cells or immune cells by inflammatory mediators can cause transcription of iNOS (NOS2) *via* NFkB[101,116]. Like eNOS and nNOS, iNOS requires Ca^{2+} for function, but the affinity is so high that iNOS is maximally activated at resting Ca^{2+} levels, and iNOS can output high levels of NO in the cell microenvironment, ranging from 10 nmol/L to μmol/L amounts[117]. These high levels of NO can be involved in immune cell killing of bacteria[117]. In contrast, nNOS and eNOS produce lower levels of NO often associated with cellular signaling pathways that regulate a variety of physiological endpoints like ciliary beating and vascular tone, as described above, and also macrophage phagocytosis[84]. However, Ca^{2+}-dependent activation of eNOS in sinonasal airway epithelial cells can directly kill bacteria like *P. aeruginosa* in the airway surface liquid[12,118]. The sinuses are thought to be sites of high NO production, important for immune function in the airways, and reduced fractional exhaled NO (F_{eNO}) is correlated with several airway diseases[10,11,119].

Beyond Ca^{2+}-calmodulin binding to eNOS, it can also be activated by Ca^{2+}-

independent mechanisms. Akt is an important regulator of eNOS function. Akt increases eNOS-mediated NO production by phosphorylation at Ser-1177 in humans and S1176 in mice[101]. Akt inhibitors such as wortmannin and LY294002 reduce NO production and PKG activity in platelets[120], while Akt co-immunoprecipitates with eNOS, suggesting the two proteins physically interact[121]. In mice *in vivo*, defective angiogenesis in Akt1 knockout mice can be rescued by a phospho-mimetic (S1176D) mutation in eNOS rendering the enzyme constitutively active[122]. This demonstrates the physiological importance of the regulation of eNOS by Akt.

Other proteins such as heat shock protein 90 (HSP90) can also associate with eNOS and modify its activity. Biochemical studies have shown a synergetic activation of eNOS by HSP90 and Akt in a calcium-independent manner in response to physiological agonist like insulin[123-126]. However, HSP90 also enhances Ca^{2+}-calmodulin activation of eNOS[124].

An important role for eNOS has been demonstrated in various models of lung injury. In the lungs of male C57BL/J6 wild-type or eNOS knockout mice exposed to mechanical ventilation, reduced phospho-Akt, phospho-eNOS, and NO leads to increased epithelial permeability. The authors concluded that the PI3K/Akt/eNOS pathway exerts significant protective effects against ventilation-induced lung injury[127]. Production of NO by eNOS may also be important for protection against neonatal hypoxia in mice[119]. Thus, data presented above suggest that activating eNOS by directly targeting PI3K/Akt signaling may have several beneficial effects in lung disease, including protection against bacterial infections, reduced damage during mechanical ventilation, or reduced inflammation.

The nuclear factor erythroid 2 related factor-2 transcription factor

Another downstream target of Akt is Nrf-2, a transcription factor that serves as a master regulator of cellular responses against oxidative stress. Nrf-2 belongs to the cap "n" collar (CNC) family of transcription factors. Nrf-2 counteracts oxidative stress and inflammation by initiating transcription of genes encoding antioxidant proteins such as NADP(H): quinone oxidoreductase (NQO1) and heme oxygenase (HO-1)[128,129]. Nrf-2 binds to a specific approximately 41 base pair consensus enhancer sequence known as the antioxidant response element (ARE) to promote transcription of antioxidant and other genes[130-132]. Nrf-2 is regulated by Kelch-like ECH- associated protein-1 (Keap1), which binds to and sequesters Nrf-2 in the cytosol and targets Nrf-2 for ubiquitination and proteasomal degradation[133,134]. Nrf-2 is rapidly turned over, with a half-life of approximately 20 min in many cells[135-137]. Keap-1 facilitates the interaction of Nrf-2 with its E3 ubiquitin ligase. However, when the Nrf-2-Keap1 interaction is disrupted, Nrf-2 can escape ubiquitination and translocate to the nucleus[134]. Disruption of the Nrf-2-Keap-1 interaction can occur by oxidative modification of cysteine thiols on Keap-1, binding of heavy metal oxidants like Cd^{2+} or Cr^{6+} to Keap-1, or activation of Akt signaling[135,138].

Activation of antioxidant gene transcription by Nrf-2 may be protective in multiple tissues against injury and inflammation in a variety of conditions such as autoimmune and neurodegenerative diseases[139,140]. Nrf-2 induction may counterbalance excess mitochondrial production of ROS, and Nrf-2 levels may be decreased in mitochondria-related neurodegenerative diseases such as Alzheimer's and Parkinson's diseases[141,142]. Nrf-2 activators are in clinical development for cancer, although, due to Nrf-2's role in promoting cell survival, there is controversy over whether activating or inhibiting Nrf-2 will be useful in different types of cancer[143,144]. In head and neck cancer, high levels of Nrf-2 may be associated with poorer patient outcomes[145]. Multiple mechanisms for aberrant activation of Nrf-2 in cancer have been reported, including Keap-1 mutations, epigenetic factors, and genetic changes[146]. Thus, while Nrf-2 is cytoprotective against oxidative stress, hyperactive Nrf-2 may be deleterious in some cancers.

Induction of Nrf-2 reduces the expression of pro-inflammatory cytokines such as IL6, IL1β, and COX2 in mice exposed to UV radiation; the same study showed that in healthy human subjects, Nrf-2 activator sulforaphane reduced solar-stimulated UV radiation-induced skin erythema[147]. Nrf-2 may interfere with lipopolysaccharide (LPS)-induced production of IL6 and IL1β in murine macrophages[148]. In the lung specifically, Nrf-2 activation may attenuate airway inflammation linked to allergy[149,150] or COPD and emphysema[151-155]. In most studies using mouse models of airway disease, the deletion of Nrf-2 results in increased inflammation and injury. Nrf-2 deficient mice are more susceptible to cigarette-smoke induced emphysema[156,157], and when cigarette-smoke-exposed Nrf-2 deficient and Wt. mice were exposed to influenza virus, Nrf-2-deficient mice exhibited higher mortality[158]. Nrf-2 may also directly modulate TLR4 signaling[159], though most studies of inflammation point to downstream effects of Nrf-2 on NF-kB-induced cytokine secretion. Nrf-2 knockout

mice exhibit more lung inflammation in response to LPS or TNFa compared with Wt. mice[9,160,161], likely *via* enhanced NF-kB signaling.

Nrf-2 is also likely important during oxidative stress induced by airway hypoxia or hyperoxia. Nrf-2-dependent reduction of alveolar growth inhibition caused by hyperoxia increases survival in newborn mice[162]. Pharmacological inhibition of Akt resulted in higher levels of inflammation and lower expression levels of antioxidant genes in mice exposed to hyperoxia, likely *via* reduced Nrf-2 signaling[163]. However, this study also found that PI3K/Akt signaling promoted inflammation after hyperoxic injury in a Nrf-2-independent manner[163]. These studies suggest that activating PI3K/Akt/Nrf-2 signaling may reduce inflammation in lung diseases where oxidative stress is an important component of the pathophysiology, though more work is needed to understand the relationship of Akt and Nrf-2 to initial injury and subsequent sustained inflammatory responses after injury.

Many experimental studies in the airway have focused on the beneficial effects of Nrf-2 activation against commonly seen oxidative stressors in lung diseases. Activation of Nrf-2 (either *via* endogenous receptors, overexpression, or activators like curcumin or sulforaphane) is protective against oxidative stress-induced lung damage caused by exposure to compounds in cigarette smoke[164-173] or H_2O_2[174]. While Nrf-2 activators have shown benefit in animal models, we hypothesize that activation of upstream PI3K/Akt signaling may also be beneficial and requires more investigation, as it would combine Nrf-2 activation with the activation of other beneficial pathways, like eNOS.

Only a limited number of studies exist on protective effects of Akt-dependent Nrf-2 activation. In prostate cancer, increased Akt and Nrf-2 activity correlated with cell survival[175]. Another study reported that raw garlic can reduce cardiac hypertrophy in fructose-fed type 2 diabetic mice through activation of the PI3K/Akt pathway; this study showed that activation of Akt increased Nrf-2 activity that protected mouse hearts from oxidative stress[176]. Similarly, overexpression of constitutively active Akt increased Nrf-2 activation in retinal pigment epithelium[177]. In this study, both the induced levels of Nrf-2 and basal levels were reduced by PI3K inhibitors wortmannin and LY294002, confirming the Nrf-2 is activated downstream of PI3K/Akt[177].

Inositol trisphosphate receptors

Inositol trisphosphate receptors (IP_3Rs) are endoplasmic reticulum (ER)-resident Ca^{2+} channels that contribute to Ca^{2+} release downstream of GPCR activation and other stimuli that activate phospholipase C[178]. Phospholipase C catalyzes hydrolysis of membrane phosphatidylinositol 4,5-bisphosphate (PIP_2) to release inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol. While diacylglycerol can activate protein kinase C, IP_3 can bind to the IP_3 receptor and sensitize it to resting cytosolic Ca^{2+} levels to cause the channel to open and promote Ca^{2+} release from endoplasmic reticulum stores[178]. The IP_3 sensitivity and Ca^{2+} release activity of IP_3Rs can be regulated by IP_3R phosphorylation by multiple kinases[179]. A consensus motif for Akt phosphorylation is contained within the C-terminal tail of all three IP_3R isoforms[180].

Phosphorylation of IP_3Rs by Akt has been suggested to reduce Ca^{2+} efflux from the ER in response to apoptotic stimuli, thus protecting cells from apoptosis[180-182]. The activity of Akt2 in lymphocytes can reduce the duration of Ca^{2+} signaling and reduce activation of the NFAT transcription factor[183,184]. However, one study suggested that the effects of Akt on IP_3Rs is specific to the type III IP_3R, while Akt activation does not affect type I IP_3R[184]. As cytokine secretion can also be driven by Ca^{2+}, reduction of Ca^{2+} signaling may reduce inflammation. However, the ability of Akt to inhibit apoptosis or inflammation may depend on the predominant subtype of IP_3R expressed in a specific cell type.

However, further research needs to be done on the role of Akt in Ca^{2+} release in airway cells. In some cells, Akt signaling may enhance Ca^{2+} release. In neurons, progesterone was shown to potentiate IP_3R-dependent Ca^{2+} release *via* Akt signaling[185,186]. Akt activity may also regulate the expression of IP_3Rs through multiple pathways. Akt2 activation of the ETS1 transcription factor may increase the expression of type II IP_3R expression in dendritic cells[187]. Additionally, Nrf-2 was shown to bind to the promoter of the gene encoding the type III IP_3R and reduce its expression in cholangiocytes, resulting in reduced Ca^{2+} signaling and reduced secretion from the bile duct[188]. Pharmacological targeting of the Akt pathway may modulate airway cell cytokine release and apoptosis through alteration of Ca^{2+} signaling, but it remains to be determined if inhibition or activation of Akt would be more beneficial.

POTENTIAL ROLES OF THE PHOSPHOINOSITIDE-3-KINASE/AKT PATHWAY IN CYSTIC FIBROSIS

Cystic fibrosis (CF) is an autosomal recessive disease caused by nearly 2000 different known mutations in the CFTR gene, which encodes the CF transmembrane conductance regulator (CFTR) protein. Although the life expectancy of CF patients is increasing with current small molecule therapies[189], CF affects approximately 75000 people in North America, Australia, and Europe[190]. The CFTR protein is expressed in the apical membranes of airway surface epithelial cells[191], airway submucosal gland serous cells[23,192,193], and a recently discovered rare cell type termed the ionocyte[194,195]. CFTR functions as chloride (Cl^-) and bicarbonate (HCO_3^-) anion channel[196] to regulate salt and fluid homeostasis and control the volume and pH of the airway surface liquid[96]. Dehydration of the ASL caused by defective CFTR function leads to thickened mucus that impairs mucociliary clearance and increases susceptibility to respiratory pathogens[31], particularly the gram-negative opportunistic bacterium *P. aeruginosa*[197]. Respiratory failure is responsible for > 95% of CF patient deaths[198]. However, the reduced flux of Cl^- and HCO_3^- ions through CFTR also affects multiple other organs where CFTR is expressed, including the exocrine pancreas, male reproductive tract, and sweat glands[31].

CFTR belongs to ATP binding cassette (ABC) superfamily of proteins and consists of two membrane-spanning domains (MSD), two nucleotide-binding domains (NBD), and a regulatory (R) domain[199]. The R domain consists of charged amino acids and several sites for phosphorylation by cAMP-dependent protein kinase A (PKA) as well as protein kinase C (PKC)[200]. Phosphorylation of the R domain enhances the association of adenosine triphosphate (ATP) to the NBDs, allowing a conformational change that results in the opening of the CFTR channel pore[201]. Subsequent hydrolysis of the ATP leads to channel closing[202]. Maturation of CFTR protein requires proper domain folding, glycosylation, and trafficking from the endoplasmic reticulum (ER) to the Golgi apparatus and eventually the plasma membrane. Dysregulation of any point in this complex multiple-step process can create a non-functional protein[203]. The most common mutation occurring in CF patients is the deletion of phenylalanine at position 508 (termed ΔF508 or F508del)[204].

In the CF lung, numerous studies have suggested the thickened mucus that is the hallmark of CF is accompanied by increased inflammation. The accumulation of neutrophils may increase inflammation and damage bronchial walls[205], while increased levels of pro-inflammatory cytokines and chemokines such as IL8 and TNF-α may also contribute to the destruction of lung tissue[206,207]. However, loss of CFTR may also confer hyper-inflammatory cellular properties, suggesting intrinsic cellular signaling defects caused by loss of CFTR function beyond the inflammation secondary to defective mucociliary clearance and bacterial infection[192,205,208-213]. CFTR itself has been linked to TLR4[214] and Akt[215,216] signaling *via* its proposed role as a signaling scaffold[217]. Thus, defective CFTR function may likely result in dysregulation of innate immunity beyond just loss of MCC[218]. All of these mechanisms may contribute to airflow obstruction, increased risk for bacterial infection, and damage to the microenvironment of the lung. It is not yet fully clear how small molecule CFTR corrector and potentiator therapies may suppress hyper-inflammation phenotypes in CF lungs[219].

Exhaled air from CF patients also contains less NO compared to non-CF individuals, possibly *via* decreased production of NO, increased metabolism of NO, downregulation of NOS enzymes, or polymorphisms in NOS genes[220-223]. Small molecule CFTR modulators have been shown to restore airway NO production, and the fraction of exhaled NO (F_{eNO}) has been proposed to be a biomarker of pharmacological restoration of CFTR[224,225]. Moreover, boosting NO signaling may also increase the effect of corrector/potentiator modulator therapies[226], suggesting multiple levels of feedback may exist between CFTR and the NO signaling pathway.

As described above, eNOS is one target of Akt. Totani and colleagues showed that inhibition of CFTR in pulmonary endothelial cells reduced NO levels *via* reducing levels of activated phosphorylated Akt and activated phosphorylated eNOS[216]. This was associated with an increase in IL8 levels. In mice, CFTR knockout macrophages had a significant reduction of Akt phosphorylation at S473 compared with control mice; this same study showed Celecoxib, an FDA-approved COX-2 inhibitor for osteoarthritis, activated the PI3K/Akt pathway and reduced inflammation in this mouse model[227]. Thus, directly targeting Akt using small molecule activators or activating upstream PI3K may enhance NO production in CF lungs and alleviate inflammation. It may also have anti-bacterial effects similar to the activation of T2R

bitter taste receptors, which drive eNOS-mediated NO production *via* Ca^{2+} rather than Akt. Of note, *P. aeruginosa*, the most common pathogen in CF lungs, is more susceptible to NO-induced killing than some other airway bacteria like *Staphylococcus aureus*[118]. A lack of efficient NO production in CF cells, possibly due to intrinsic defects in Akt signaling, may partly contribute to why these bacteria are so prevalent in CF lungs while almost never causing infection in non-CF patients unless non-CF patients are otherwise immunocompromised[228].

NO itself has been suggested to activate CFTR *via* PKG in some studies[229-231], while NO has also been reported in other studies to have no effect on CFTR[232], inhibit CFTR trafficking and/or activation[233,234], or activate non-CFTR Cl^- currents[235]. Part of the discrepancy may be that most studies use different NO donor compounds at different concentrations, as well as occasionally more physiological ways to induce NO production (*e.g.*, receptor activation). While no one has thoroughly examined the activation of CFTR downstream of specific Akt activation in the airway, targeting Akt would increase NO production to a more physiological level than NO donor compounds. Akt activation would stimulate endogenous eNOS, the major NOS isotype in uninflamed airway cells[114].

As indicated earlier, Nrf-2 is also a downstream target of Akt that plays a cytoprotective role against oxidative stress. Nrf-2 may convey resistance to pyocyanin, a bacterial product from *P. aeruginosa* that causes oxidative stress. The PI3K/Akt pathway is activated in lung epithelial cells during pyocyanin exposure, and the increased transcription of antioxidants may protect these cells from death[236]. It has been suggested that defective Nrf-2 in CF cells causes enhanced oxidative stress that increases inflammatory cytokine production[237], and Nrf-2 function is restored when mutant CFTR function is enhanced by small molecule therapeutics[238]. Alterations of Nrf-2 signaling in CF may also be tied to alterations in cAMP signaling and the CREB binding protein[239]. Boosting Nrf-2 function by targeting the PI3K/Akt pathway may have beneficial effects in CF lungs.

Furthermore, Nrf-2 may also regulate expression of CFTR itself[240]. We hypothesize that a direct Nrf-2 activator such as curcumin[241], dimethyl fumarate[242] or andrographolide[165] and/or activating Nrf-2 *via* Akt may be useful in combination with small molecule CFTR correctors and potentiators[189]. Such a strategy may further increase the number of functional CFTR channels at the plasma membrane by boosting CFTR gene transcription. This may be useful in patients where specific CFTR mutations reduce the efficiency of small molecule correction or potentiation.

CONCLUSION

The respiratory epithelia are in constant contact with bacteria, viruses, and pathogens during every breath. Airway innate immunity is the first line of host defense against these challenges[243]. Some of the strategies of the innate immune system of the airways include mucociliary-clearance, antimicrobial peptide secretion, NO production, cytokine secretion, and antioxidant gene production (Figure 3)[244]. PI3K/Akt signaling is one of the major signaling pathways regulating multiple components of these processes. Akt signaling maybe altered in airway diseases like CF. Together, the above studies discussed in this review suggest that therapeutic strategies to enhance the PI3K/Akt pathway and increase NO production, boost antioxidant transcription *via* Nrf-2, or activate other anti-inflammatory pathways might be particularly beneficial in CF patients. These strategies may also benefit patients with other inflammatory airway diseases like CRS, asthma, and/or COPD. Because pharmacological tools to inhibit PI3K[245,246], inhibit Akt[247-249], or even directly activate Akt[77] are available, exploring the effects of Akt signaling in airway cells may yield druggable targets that can be translated to human therapeutics.

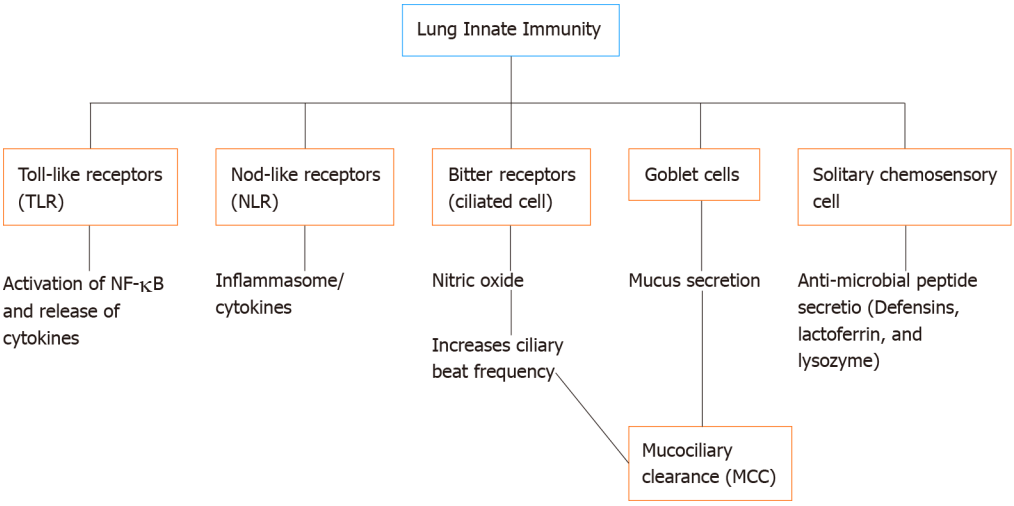

Figure 3 Summarization of non-specific immune strategies in lung innate immunity. Toll-like receptors are activated to recognize pathogen-associated microbial patterns/damage-associated molecular pattern (PAMPs/DAMPs) and activate nuclear factor-kB to produce cytokines; nod-like receptors are cytosolic receptors that can recognize intracellular PAMPs/DAMPs and can form inflammasome to either produce cytokines such as interferons or induce pyroptosis (inflammation associated apoptosis); Stimulation of bitter receptors, expressed in ciliated cells in the airways, elevates Ca^{2+} that produces NO which activates protein kinase G and increase ciliary beat frequency; NO can directly kill bacteria; Goblet cells produce mucus that traps pathogens and bacteria which are then eliminated out of the upper airway system through MCC; Activation of bitter receptors in solitary chemosensory cells can increase intracellular Ca^{2+} that can produce antimicrobial peptides.

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REVIEW

Regulation of cytochrome c oxidase contributes to health and optimal life

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Abstract

The generation of cellular energy in the form of ATP occurs mainly in mitochondria by oxidative phosphorylation. Cytochrome c oxidase (CytOx), the oxygen accepting and rate-limiting step of the respiratory chain, regulates the supply of variable ATP demands in cells by "allosteric ATP-inhibition of CytOx." This mechanism is based on inhibition of oxygen uptake of CytOx at high ATP/ADP ratios and low ferrocytochrome c concentrations in the mitochondrial matrix *via* cooperative interaction of the two substrate binding sites in dimeric CytOx. The mechanism keeps mitochondrial membrane potential $\Delta\Psi_m$ and reactive oxygen species (ROS) formation at low healthy values. Stress signals increase cytosolic calcium leading to Ca^{2+}-dependent dephosphorylation of CytOx subunit I at the cytosolic side accompanied by switching off the allosteric ATP-inhibition and monomerization of CytOx. This is followed by increase of $\Delta\Psi_m$ and formation of ROS. A hypothesis is presented suggesting a dynamic change of binding of NDUFA4, originally identified as a subunit of complex I, between monomeric CytOx (active state with high $\Delta\Psi_m$, high ROS and low efficiency) and complex I (resting state with low $\Delta\Psi_m$, low ROS and high efficiency).

Key Words: Cytochrome c oxidase; Regulation of respiration; Allosteric ATP-inhibition; NDUFA4; Reversible phosphorylation; Efficiency of ATP synthesis; Dimerization of cytochrome c oxidase

Core Tip: This article describes the "allosteric ATP-inhibition of cytochrome c oxidase," which prevents the formation of reactive oxygen species (ROS) under resting conditions in all eukaryotic cells by keeping the mitochondrial membrane potential ΔΨm at low values. Under stress – *via* increased calcium concentrations – this mechanism is switched off, accompanied by increased rates of ATP-synthesis with decreased efficiency and formation

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of deleterious ROS. A hypothesis is described in which NDUFA4 changes its position from complex I to cytochrome c oxidase when the metabolic state changes from the rest to excited state under stress.

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INTRODUCTION

All expressions of life require energy in the form of ATP, the universal energy currency of living cells. Large variations in ATP turnover with rates up to 100-fold in skeletal muscle[1,2] occur in cells depending on the tissue. ATP is mostly produced in mitochondria by oxidative phosphorylation. In four enzyme complexes of the respiratory chain in the inner membrane of mitochondria, electrons from NADH and $FADH_2$, the reduced equivalents of nutrients, are successively transferred via the final complex IV = cytochrome c oxidase (CytOx) to molecular oxygen (O_2), forming water in a strongly exergonic reaction. In three complexes, complex I (NADH dehydrogenase), complex III (ubiquinol: Cytochrome *c* oxidoreductase) and complex IV, the energy of this "cold combustion" of nutrients is released in an electrochemical proton gradient $\Delta\mu H^+$ across the membrane. Peter Mitchell described the energy of $\Delta\mu H^+$ as proton motive force $\Delta p = \Delta\mu H^+/F$ (F = Faraday constant), consisting of an electrical ($\Delta\Psi_m$) and a chemical part (ΔpH_m): $\Delta p = \Delta\Psi_m - 59\Delta pH_m$ (mV)[3]. However, most of the proton motive force in mitochondria consists of $\Delta\Psi_m$ and reaches values of 180-200 mV in isolated mitochondria at state 4 (controlled state of respiration at limited ADP)[4,5]. The energy of $\Delta\mu H^+$ is used by the ATP synthase (complex V), *via* backward flow of protons, to drive the endergonic reaction: ADP + phosphate → ATP. In the active state 3 of isolated mitochondria (presence of ADP), $\Delta\Psi_m$ is lower (130-140 mV), since it is partly consumed by the ATP synthase.

REACTIVE OXYGEN SPECIES IN MITOCHONDRIA

Health and optimal life are frequently hurt by the consequences of psychosocial stress. The consequences appear in cells as "oxidative stress" caused by the over-production of reactive oxygen species (ROS, mainly $O_2^{\cdot-}$ and H_2O_2) in mitochondria. While low amounts of ROS have in cells signaling functions[6,7], high amounts produced in mitochondria are generally assumed to participate in aging[8-10] and in the generation of numerous diseases including cancer, hypertension, atherosclerosis, ischemia /reperfusion injury, neurodegenerative diseases like Alzheimer's and Parkinson's disease, rheumatoid arthritis, diabetes mellitus, and mitochondrial diseases[11-14].

It was found that ROS are generated in the respiratory chain at increasing $\Delta\Psi_m$-values above 130-140 mV[15-17]. The superoxide radical anion $O_2^{\cdot-}$ is mostly produced at complexes I and III, due to the transfer of a single electron to O_2[18,19], and is immediately converted into H_2O_2 by mitochondrial superoxide dismutases[20].

In most situations of animals, *e.g.*, during sleep, the resting state predominates with high ATP-levels, low amounts of ADP, and low consumption of ATP (at least in skeletal muscles). According to the results with isolated mitochondria at these low rates of ATP consumption (state 4, with $\Delta\Psi_m$ values of 180-200 mV[4,5]), large amounts of ROS would be produced under resting conditions. Fortunately in resting living cells, mitochondrial $\Delta\Psi_m$ values are low, between 100 and 130 mV (for references see[21]). These low $\Delta\Psi_m$ values are sufficient for maximal rates of ATP synthesis, since the rate of ATP synthesis by the ATP synthase is saturated and maximal at 100-120 mV[22]. But how are these low $\Delta\Psi_m$ values of 100-130 mV achieved to maintain a healthy life?

It was found, however, that under various stress conditions a transient increase of the mitochondrial membrane potential does occur, called "hyperpolarization," which in some cases is followed by cell apoptosis[23]. Both, the low $\Delta\Psi_m$ values of 100-130 mV in resting living cells and the hyperpolarization of $\Delta\Psi_m$ under stress are explained below by the "allosteric ATP-inhibition of CytOx."

CYTOCHROME C OXIDASE, A CONTROLLING POINT OF OXIDATIVE METABOLISM

CytOx developed early during evolution as the final oxygen accepting enzyme of respiratory chains for the generation of ATP by oxidative phosphorylation[24]. With increasing organismal complexity during evolution the number of protein subunits in the CytOx complex increased from 2-3 in bacteria over 7 in the slime mold *Dictiostelium discoideum*, and 11 in yeast to 13 in mammals[25]. In eukaryotes the "catalytic" subunits I-III are encoded on mitochondrial DNA and synthesized in mitochondria. The additional "supernumerary" subunits are encoded by the nuclear DNA and synthesized on cytoplasmic ribosomes. A complicated machinery is required for the transport of these subunits into mitochondria[26] and for the assembly into the 13-subunit CytOx complex of vertebrates[27].

In contrast to many other "oxidases"[28], CytOx produces no ROS during reduction of dioxygen, due to its unique binding site for O_2 in subunit I, composed of heme a_3, Cu_B and a tyrosyl-group, allowing simultaneous transfer of 4 electrons to O_2[29]. The binding site for cytochrome *c* containing two copper atoms is located in subunit II, and subunit three stabilizes the core subunits. The catalytic center of CytOx, located in subunits I-III, is very similar in bacteria and in eukaryotes, and the basic functions, *i.e.* reduction of oxygen[30] and generation of an electrochemical potential $\Delta\mu H^+$[31] are the same. Therefore the role of "supernumerary" subunits in the activity of CytOx was ignored. In the fourth edition of their textbook "Bioenergetics4"[32], Nicholls and Ferguson denied the catalytic function of the supernumerary subunits. However, by the use of subunit-specific antibodies for 3 of the ten nuclear encoded subunits a specific function on the activity of CytOx was demonstrated. In subunit IV: The "allosteric ATP-inhibition" *via* binding of ATP at its matrix domain at high ATP/ADP-ratios[33], and also in subunit IV: The decrease of cytochrome *c* affinity by binding ATP to the intermembrane domain at high ATP/ADP-ratios[34]. In subunit Va: The abolishment of the "allosteric ATP-inhibition" by binding of 3,5-diiodothyronine[35], and in subunit VIa-heart isoform: The decrease of H^+/e^--stoichiometry from 1 to 0.5 at high ATP/ADP-ratios[36].

From application of the metabolic control analysis to isolated mitochondria[37-39] a 5- to 7-fold excess of CytOx capacity was found over the amount required to support the endogenous respiration of mitochondria[40-42]. However, later studies with intact cells demonstrated that CytOx represents the rate limiting step of oxidative phosphorylation in living cells[43,44].

FEEDBACK INHIBITION OF CYTOX BY ATP: THE "ALLOSTERIC ATP-INHIBITION OF CYTOX"

The "allosteric ATP-inhibition of CytOx" based on the exchange of bound ADP by ATP at the matrix domain of CytOx subunit IV-1 at high ATP/ADP ratios originally discovered in 1997[32,45], represents a feedback inhibition of mitochondrial respiration by its final product ATP. We have described this mechanism in more than 20 publications and discussed its implications on human health more recently[46-48].

The bovine heart enzyme contains 10 high-affinity binding sites for ADP seven of which are exchanged by ATP at high ATP/ADP ratios[34]. The exchange of bound ADP by ATP at high ATP/ADP-ratios (half-maximal at ATP/ADP = 28) induces a sigmoidal inhibition curve in the kinetics of oxygen uptake *vs* ferrocytochrome *c* concentration (Hill-coefficient = 2[45]). This kinetic behaviour indicates a cooperativity between two binding sites of the substrate ferrocytochrome *c*. Since the CytOx monomer contains only one binding site for cytochrome C[49], a dimeric CytOx structure is required for the feedback inhibition of CytOx activity by the "allosteric effector" ATP. At lower ATP/ADP-ratios the CytOx kinetics exhibits normal hyperbolic saturation curves. The allosteric ATP-inhibition is independent of $\Delta\Psi_m$[45]. The ATP/ADP ratio in the mitochondrial matrix for half-maximal inhibition of CytOx activity at ATP/ADP = 28[45] corresponds to the high cytosolic ATP/ADP ratio of 100-1000 determined by ^{31}P-NMR measurements in rat heart[50]. Due to $\Delta\Psi_m$ the ATP/ADP-ratio in the mitochondrial matrix will be lower (ATP/ADP = 4-40, see[47]).

The first crystal structure of CytOx was a dimer[51]. But the structure of the physiological relevant CytOx dimer must be slightly different because in the crystals 10 molecules of cholate are bound per CytOx monomer[52]. The exchange of cholate by ADP in the cholate-CytOx is a slow process and accompanied by a spectral change[52].

This contrasts with the immediate exchange of bound ATP by ADP in the ADP-CytOx[53] which indicates the non-physiological structure of the cholate-CytOx crystals[51]. In fact, the crystallisation of the native ADP-CytOx or ATP-CytOx appears not possible. Only by using cholate enough CytOx could be obtained for crystallization (Kyoko Shinzawa-Itoh, personal communication). The control of respiration by the allosteric ATP-inhibition of CytOx, also named "second mechanism of respiratory control"[54], is independent of $\Delta\Psi_m$[45], in contrast to the classical "respiratory control" where mitochondrial respiration is limited at high $\Delta\Psi_m$ values[5].

The allosteric ATP-inhibition of CytOx keeps $\Delta\Psi_m$ at low values (< 130 mV), due to feedback inhibition of CytOx activity by ATP at high ATP/ADP-ratios, preventing further increase of $\Delta\Psi_m$ by proton pumping within complexes I, III, and IV of the respiratory chain. The inhibitory effect of ATP on $\Delta\Psi_m$ has also been measured directly in isolated rat liver mitochondria using a tetraphenyl phosphonium electrode[55]. The low ROS production in mitochondria of living cells under resting conditions[18] is thus explained by the allosteric ATP-inhibition of CytOx which maintains low $\Delta\Psi_m$ values[46]. Therefore, this mechanism contributes to the health and optimal life of higher organisms.

It was suggested that the allosteric ATP-inhibition of CytOx contributes to an optimal efficiency of oxidative phosphorylation and is switched off under stress and excessive work in order to increase the rate of ATP synthesis which is accompanied by lower efficiency[47]. Furthermore, it was assumed that higher efficiency may be achieved by increased H^+/e^--stoichiometry of proton pumping in CytOx[46]. In general, a constant $H^+/e^- = 1$ was assumed for CytOx[56,57]. The Yoshikawa group identified in bovine heart CytOx a third proton channel, the H-channel[58-61] which is absent in bacterial CytOx[62]. We suggested that the allosteric ATP-inhibition which maintains low $\Delta\Psi_m$ values could increase the H^+/e^--stoichiometry of CytOx to 2, based on additional proton pumping through the H-channel which is energetically possible[47]. In fact, a H^+/e^--stoichiometry of 2 was previously measured for CytOx in isolated rat liver mitochondria[63-66].

In bovine heart mitochondria, most CytOx (> 85%) occurs as free complexes[67] not assembled into supercomplexes like respirasomes[68]. In the respirasome $I_1III_2IV_1$ CytOx appears as monomer[69,70] where the binding site between the two monomers in the dimeric crystal structure[51,71] is free and allows dimerization of two respirasomes. This holds also for the megacomplex $I_2III_2IV_2$[72].

The allosteric ATP-inhibition of CytOx is active in most cell types which express subunit IV-1. The isoform subunit IV-2 was found to be expressed in human cell lines under hypoxia[73]. Also in isolated astrocytes and cerebellar granule cells subunit IV-2 is expressed under hypoxic conditions accompanied by an abolition of the allosteric inhibition of CytOx by ATP[74].

STRESS TURNS OFF THE ALLOSTERIC ATP-INHIBITION OF CYTOX *VIA* CYTOSOLIC CALCIUM

The fact that the feedback inhibition of CytOx by ATP has been ignored for more than 15 years is also based on its unique biochemical properties. It was found to be dependent on phosphorylation of CytOx subunit I at the cytosolic side. After dephosphorylation of this site by a calcium-activated protein phosphatase (PP1) the allosteric ATP-inhibition of CytOx is switched off. Rephosphorylation by a cAMP-dependent protein kinase (PKA) switches it on again. These observations were made with the isolated enzyme which was partly reconstituted in liposomes[75-77]. Recently these properties could also be shown with intact rat heart mitochondria. In this study a very low concentration of calcium (1-10 micromolar) was sufficient to switch off the allosteric ATP-inhibition[1]. Various stress signals, including psychosocial stress, increase the cytosolic calcium concentration and activate a Ca^{2+}-dependent protein phosphatase which is located in the mitochondrial intermembrane space leading to dephosphorylation of CytOx at the cytosolic side of subunit I[76]. This dephosphorylation is accompanied by loss of the allosteric ATP-inhibition, an increase of $\Delta\Psi_m$ and ROS formation[48].

In conclusion, the "allosteric ATP-inhibition of CytOx" has four physiological functions: (1) To maintain a constant high ATP/ADP ratio in cells; (2) To inhibit the oxygen consumption of mitochondria when sufficient ATP is available; (3) To prevent the formation of ROS under resting conditions by keeping the mitochondrial membrane potential $\Delta\Psi_m$ at low values; and (4) To increase the rate of respiration and ATP synthesis during excessive workload or stress by switching it off. This is

accompanied by reduced efficiency and generation of deleterious ROS.

NDUFA4, A RESPIRATORY CHAIN-ASSOCIATED FACTOR

NDUFA4 was identified as a nuclear-encoded subunit of complex I[78-80]. However, together with two other subunits its gene had significantly increased amino acid substitution rates during primate radiation, suggesting that they have been subjected to adaptive selection[81]. Later, NDUFA4 was no longer considered a subunit of complex I[82]. Recently NDUFA4 was claimed to represent the 14th subunit of mammalian CytOx[83-86]. The cryo-EM structure of a NDUFA4-CytOx complex could be determined, where NDUFA4 is bound to the CytOx monomer exactly at the binding site between the two monomers in the dimeric enzyme[87]. If NDUFA4 would represent an essential subunit of CytOx, a dimeric structure, as determined in CytOx crystals by Tsukihara *et al*[51], would be impossible. We doubted the claim that NDUFA4 represents the 14th subunit of mammalian CytOx. This doubt is based on immunoprecipitation of the 13-subunit CytOx from Triton X-100 dissolved rat liver mitochondria[88]. In addition, the feedback inhibition of CytOx by ATP *via* cooperativity of two binding sites for cytochrome *c* in the dimeric enzyme (the allosteric ATP-inhibition of CytOx), would be impossible with the monomeric NDUFA4-CytOx complex. Recently we concluded from studies with intact isolated rat heart mitochondria[1] that cAMP-dependent phosphorylation at the intermembrane side of CytOx subunit I[76] induces a dimeric enzyme with allosteric ATP-inhibition, while calcium-activated dephosphorylation monomerizes CytOx accompanied by abolishment of the allosteric ATP-inhibition and binding of NDUFA4[87]. These results strongly suggest that stress-dependent increase of cytosolic calcium leads to a rise of $\Delta\Psi_m$ and ROS formation at lower efficiency due to loss of the allosteric ATP-inhibition of CytOx[47].

HYPOTHESIS

The following hypothesis describes a dynamic change of reversible protein-protein interactions which are not expected from X-ray crystal structures or cryo-EM structures but occur frequently in cells (see *e.g.*,[89]).

We postulate that NDUFA4 (N-terminal amino acid sequence: MLRQ) changes its binding position between complex I and CytOx, depending on the stress situation and/or energetic (ATP) requirements of cells (Figure 1). We postulate that increased cytosolic calcium concentration (> 1 micromolar), as a consequence of various stress factors, activates a calcium-dependent protein phosphatase in the intermembrane space of mitochondria, which dephosphorylates the CytOx dimer, accompanied by monomerization of CytOx, loss of the allosteric ATP-inhibition, and binding of NDUFA4. Also complex I is postulated to be dephosphorylated by a calcium-dependent protein phosphatase, accompanied by decreased affinity of complex I to NDUFA4 which binds to monomeric COX forming the NDUFA4-CytOx complex[87]. The binding of NDUFA4 to phosphorylated complex I and its dissociation after dephosphorylation could be tested by BN-PAGE with mitochondria either treated with cAMP or with calcium (see[1]), followed by immunodetection with specific antibodies. The physiological function of binding NDUFA4 to (phosphorylated) complex I under relaxed conditions could be to decrease the affinity of complex I to NADH because high NADH/NAD$^+$-ratios were shown to stimulate ROS production in complex I[90-93].

Various stress signals were shown to increase cytosolic calcium concentrations in cells including high glutamate[94] or glucose[95]. In addition, psychosocial stress was shown to increase cytosolic calcium, as shown in cardiomyocytes[96], platelets[97], hippocampal-derived HT22 cells[98], urothelial cells[99], and cardiomyocytes[100]. Under resting conditions the cytosolic calcium concentration is low (about 0.1 micromolar), and a cAMP-dependent PKA rephosphorylates CytOx, accompanied by dimerization and switching on the allosteric ATP-inhibition of CytOx. This was shown in previous in vitro studies with the isolated CytOx by phosphorylation with cAMP-dependent PKA+ATP. In addition it was switched off by dephosphorylation with a calcium-activated PP1[76]. Dimerization of CytOx is possible between single CytOx complexes as well as between respirasomes since the binding domain between monomers in dimeric CytOx[71] is free in supercomplexes[70,72].

The function of binding NDUFA4 to monomeric CytOx is still unclear. The change

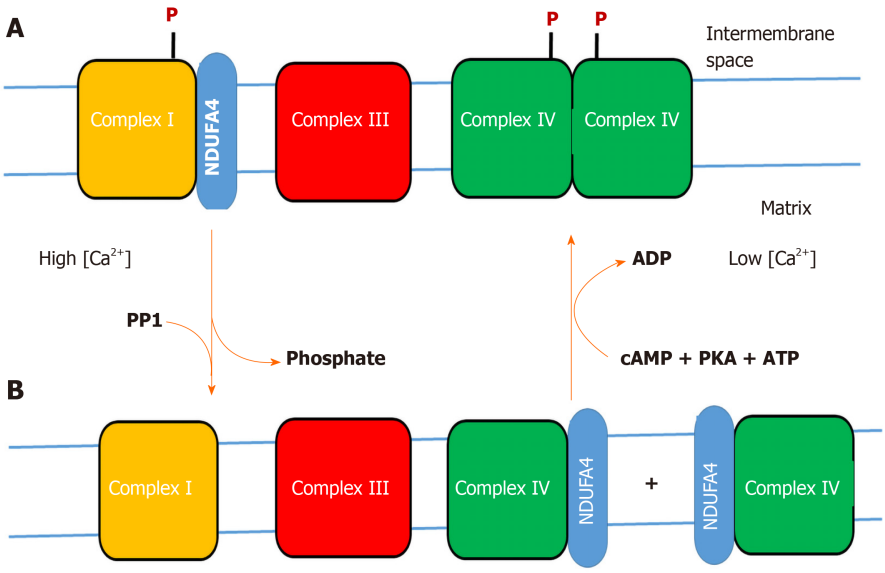

Figure 1 Hypothesis on the variable binding of NDUFA4 to complex I or cytochrome c oxidase. A: Phosphorylation (P) of complex I and cytochrome c oxidase (CytOx) at low cytosolic calcium (< 1 micromolar) stabilizes binding of NDUFA4 to complex I and the function of the "allosteric ATP-inhibition" of dimeric CytOx (resting state); B: Stress-induced increase of cytosolic calcium (> 1 micromolar) dephosphorylates complex I and CytOx by a calcium-activated PP1, accompanied by monomerization of CytOx and switching off its allosteric ATP-inhibition. NDUFA4 is detached from complex I and binds to monomeric CytOx (excited state). At low cytosolic calcium, a cAMP-dependent protein kinase A phosphorylates complex I and CytOx. This changes the binding position of NDUFA4 from CytOx to complex I, accompanied by dimerization of CytOx and activation of its allosteric ATP-inhibition.

of dimeric CytOx to monomeric CytOx (NDUFA4-CytOx) is associated with the loss of allosteric ATP-inhibition and increase of the rate of respiration and ATP synthesis at lower efficiency. The dimeric enzyme composed of two phosphorylated 13-subunit monomers represents CytOx in the resting state with higher efficiency (probably with increased H^+/e^- -stoichiometry = 2)[47]. Our view is different from that of Shinzawa-Itoh *et al*[101] who described the dimeric CytOx as the physiological standby form in the mitochondrial membrane.

The role of NDUFA4 as 14th subunit of CytOx was suggested by[84] based on mutations in the *NDUFA4* gene accompanied by defective CytOx activity in patients with Leigh syndrome. In muscle tissue from patients the NDUFA4 protein was absent while the CytOx complex was still there but without activity. Since the literature is full of papers measuring CytOx activity with the isolated 13-subunit enzyme (without NDUFA4), the physiological function of NDUFA4 remains unknown. We suggest to rename it to "mitochondrial respiratory chain associated factor 1".

CONCLUSION

A hypothesis is presented suggesting a dynamic change of binding of NDUFA4, originally identified as a subunit of complex I, between monomeric CytOx (active state with high $\Delta\Psi_m$, high ROS and low efficiency) and complex I (resting state with low $\Delta\Psi_m$, low ROS and high efficiency).

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MINIREVIEWS

Epigenetic basis of Alzheimer disease

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Abstract

Alzheimer disease (AD) is the primary form of dementia that occurs spontaneously in older adults. Interestingly, the epigenetic profile of the cells forming the central nervous system changes during aging and may contribute to the progression of some neurodegenerative diseases such as AD. In this review, we present general insights into relevant epigenetic mechanisms and their relationship with aging and AD. The data suggest that some epigenetic changes during aging could be utilized as biomarkers and target molecules for the prevention and control of AD.

Key Words: Epigenetics; Aging; Neurodegenerative diseases; Alzheimer disease

Core Tip: The deregulation of non-coding ribonucleic acids and epigenetic modifications have been described in Alzheimer disease (AD). These changes have been observed in different brain regions related to learning and memory, processes that are affected in AD. The epigenetic basics in the progression of AD were integrated into this review.

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INTRODUCTION

Alzheimer disease (AD) is the most common form of neurodegenerative dementia. AD is characterized by memory loss and rapidly progresses to symptoms such as personality changes and language problems, leading to a loss of the ability to perform routine activities and eventual death of the individual. Diagnosed AD progresses over approximately 8-10 years, but the first events of this disease can occur up to 20 years beforehand. Of all AD cases, more than 95% occur sporadically in adults aged 65 years or older, defined as late-onset AD. Multiple factors, such as environmental, biological, and genetic susceptibility, are associated with the development of AD. Less than 1% of AD cases are related to genetic mutations; these cases generally occur in younger adults (approximately 45 years old). Furthermore, some polymorphisms have been implicated in the development of AD; for instance, the apolipoprotein E 4 (*APOE*4) variant is associated with an increase of risk to develop AD, while *APOE*2 variant seems to decrease this risk[1-4].

The development and progression of AD are linked to the dysfunction and death of neurons, which generally appear to originate in the hippocampus, frontal and temporal lobes, and the limbic system, extending to neocortex regions as the disease progresses. In turn, these events are linked to the detection of two neuropathological structures: β-amyloid plaques and neurofibrillary tangles (NFTs) (Figure 1).

The primary component of β-amyloid plaques is the β-amyloid peptide, which is generated and secreted *via* proteolysis of the β-amyloid precursor protein (*APP*) by enzyme complexes known as β-secretases (*BACE*) and γ-secretases, which contain presenilin 1 (PS1, encoded by *PSEN1*) and PS2 (encoded by *PSEN2*) as subunits[5]. β-amyloid peptides have lengths ranging from 38 to 43 amino acids and are generated in neurons and released in the extracellular space[6]. Although their function is unclear, they may play a role in synaptic plasticity. Under normal conditions, β-amyloid peptide is eliminated *via* several mechanisms such as: (1) Ubiquitin-proteasome system; (2) Autophagy-lysosome system; (3) Proteases; (4) Microglial or astrocytic phagocytosis; and (5) Blood circulatory clearance[1,7]. Additionally, it has been suggested that *APOE* expression improves β-amyloid clearance[8]. However, these mechanisms may deteriorate upon aging and may thus contribute to the accumulation of β-amyloid peptides, forming neurotoxic plaques. These amyloidogenic plaques are surrounded by glial cells and are associated with dystrophic neurites (random neuron prolongations caused by the accumulation of abnormal filaments); therefore, these plaques lead to fibrillar degeneration in nerve cells. The degree of AD is apparently correlated with the proportion of β-amyloid plaques leading to neurodegeneration[9-11].

NFTs are formed by paired helical filaments comprising dense accumulations of insoluble polymers, with the hyperphosphorylated tau protein as the primary component. The tau protein is a 50-64 kDa thermostable protein associated with tubulin. Tau promotes the assembly of microtubules in the neuron cytoskeleton, and the tau-phosphorylation regulates this function. NFTs are formed in the perinuclear region of hippocampal neurons, and according to analyses of postmortem samples from individuals with AD, the quantity of NFTs correlates with the severity of AD. It has been proposed that β-amyloid plaques can also promote intracellular tau aggregation. Additionally, the release of tau damages other cells. Thus, AD is characterized by the presence of extracellular plaques containing insoluble β-amyloid filament accumulations, NFTs formed by hyperphosphorylated tau, and neuroinflammation. These elements are critical markers of AD and contribute to the neurotoxicity of this disease[9,12].

Currently, AD can be diagnosed in living patients by positron emission tomography (PET) and cerebrospinal fluid (CSF) techniques. In PET, a radionuclide as florbetapir (^{18}F) is used as a marker for β-amyloid, which crosses the blood–brain barrier and binds to β-amyloid fibrillar plaques with high affinity, enabling *in vivo* computerized imaging of β-amyloid plaques. Some studies have shown that deposition of these plaques begins decades before dementia, cognitive decline, and brain atrophy. To detect AD by CSF, a lumbar puncture is performed, and β-amyloid 1-42, T-tau (total), and P-tau (phosphorylated, for example, pThr181) are assessed as biomarkers. A positive β-amyloid PET and CSF detection of low levels of β-amyloid 1-42 and high levels of T-tau or P-tau are criteria for the diagnosis of AD[1,6].

FOUNDATIONS OF EPIGENETICS

Chromatin consists of deoxyribonucleic acid (DNA) mainly associated with histone

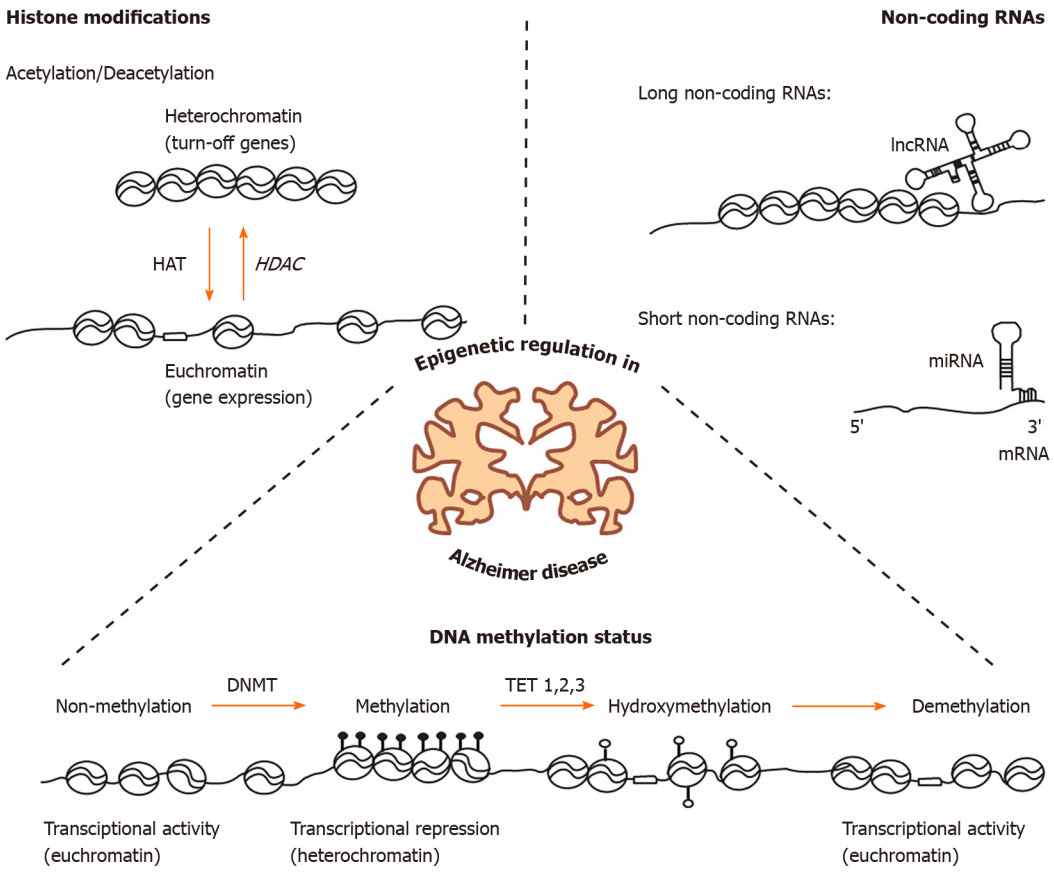

Figure 1 Epigenetics modifications associated with Alzheimer disease. Acetylation and de-acetylation of histones (left), deoxyribonucleic acid methylation status (middle, bottom) and the expression of non-coding ribonucleic acid (RNA) [short (miRNAs) and long (lncRNAs)] (right) are altered in Alzheimer disease. HDAC: Histone deacetyltransferase; DNA: Deoxyribonucleic acid; RNA: Ribonucleic acid; mRNA: Messenger RNA; miRNA: MicroRNA; lncRNA: Long non-coding RNAs.

proteins; an octamer of histones surrounded by 147 base pairs of DNA forms the nucleosome, which is the basic unit of chromatin. Epigenetics refers to chromatin structure changes that affect gene expression. The conformation of chromatin is highly dynamic, oscillating from an open, lax, or relaxed state to a compact, non-relaxed, and closed state and vice versa. A compact chromatin structure inhibits transcription, while the relaxed form of chromatin promotes this process. Conformational changes in chromatin are orchestrated by the action of co-regulatory proteins, which are divided into co-repressors and co-activators. Co-regulators are proteins that do not bind to DNA directly, but through their interaction with transcription factors. Importantly, co-regulators may have an enzymatic activity to modify chromatin and/or recruit other co-regulatory proteins with the catalytic ability to produce these changes in chromatin structure[13,14].

It has been demonstrated that epigenetic regulatory events involve histone acetyltransferase and histone deacetyltransferase (HDAC) enzymes. For example, some co-activator complexes exhibit histone acetyltransferase activity, in which an acetyl group is added to the lysines at the N-terminus of histones to neutralize their positive charge, thus weakening their strong interaction with DNA and relaxing the chromatin to promote gene expression. In contrast, co-repressor complexes with HDAC activity remove the acetyl group and promote chromatin compaction to inhibit gene expression[13]. In addition to acetylation/deacetylation, histones also undergo other modifications by adding or removing other functional groups (*e.g.*, methylation or phosphorylation) or small proteins (*e.g.*, ubiquitination or sumoylation), which affects the chromatin conformation. It has been suggested that the combination of modifications generates a histone code for chromatin restructuring, which regulates gene expression[13,15].

Another epigenetic modification occurs in the cytosine residues of the CpG dinucleotide of DNA through the addition of a methyl group by DNA methyltransferases. This modification affects the binding of transcription factors to consensus sites, and recruits methylated DNA-binding proteins that bind to co-

repressors and HDACs, compacting the chromatin and inhibiting gene expression[16]. Active DNA demethylation is mediated by the methylcytosine dioxygenase enzyme known as ten-eleven translocation, which oxidizes 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC), followed by DNA repair to generate an unmodified cytosine. Therefore, DNA methylation is detected by 5-mC marks and is associated with transcriptional repression, while DNA with 5-hmC marks is related to co-activator recruitment and transcriptional activity[17,18]. Moreover, ATP-dependent chromatin remodelers displace DNA and reposition it within nucleosomes, creating areas of DNA with access to transcriptional machinery. It is worth noting that epigenetic modifications are linked, as there are proteins that modify chromatin and others that can read these modifications, generating a dynamic system coordinated by several marks and regulatory complexes that modulate gene expression[14].

Other molecules involved in epigenetics include non-coding ribonucleic acids (RNAs), which can be small [less than 200 nucleotides (nt) or long (more than 200 nt)[19,20]. MicroRNAs (miRNAs) are small non-coding RNAs with a 19-25 nt hairpin structure that can imperfectly match (partial complementarity with 6-8 nt) to the sequence of messenger RNA (mRNA), resulting in inhibition of its translation or mRNA degradation[21]. Genes located in active chromatin regions appear to be more commonly regulated by miRNAs[22]. In contrast, long non-coding RNAs (lncRNA) regulate the chromatin structure by interacting with proteins, RNA, and DNA[23-25]. Therefore, lncRNAs can act as hooks for proteins or miRNAs, competitive inhibitors of molecular interactions, scaffolding to bring proteins closer for interaction, guides for binding of protein complexes, and gene transcription activators by favoring promoter–enhancer interactions[23-25].

NEURODEGENERATIVE DISEASES, AGING, AND EPIGENETICS

Neurodegenerative diseases are caused by the degeneration, dysfunction, and irreversible death of neurons in specific regions of the central nervous system. In this system, glial cells that include astrocytes, microglia, and oligodendrocytes, are important for the support and proper functioning of neural connections, and these are also affected in neurodegenerative diseases[26]. AD (which affects memory), Parkinson's disease, and amyotrophic lateral sclerosis (which affects motor activities) are some examples of neurodegenerative diseases[27]. These diseases frequently manifest in older adults; thus, the first risk factor for their development is aging, which has been associated with processes of cellular senescence. Senescence is a viable and metabolically active state of cells, but it is also a non-proliferative state accompanied by pro-inflammatory secretory activity. Senescence is a mechanism for controlling damaged cells, but the accumulation of senescent cells during aging competes with normal cells, blocking the capacity for tissue regeneration and releasing factors that stimulate chronic inflammation and contribute to neuronal degeneration[27-29].

During senescence, epigenetic changes are detected, such as the formation of senescence-associated heterochromatic foci, *i.e.*, compacted chromatin linked to a reduced expression of histone-encoding genes. However, senescent cells also show a lower number of repressive heterochromatin marks such as DNA methylation and histone methylation (H3K9me3, H3K27me3, and H4K20me3), suggesting an increased expression of many other genes[30,31]. Taken together, epigenetic modifications lead to drastic changes in the pattern of gene deactivation and activation in cellular senescence, which, when enriched in cells of the nervous system during aging, may lead to the development of neurodegeneration[30,31].

During aging, epigenetic changes related to acetylation processes can be detected; for example, the H4K16ac mark has been associated with a state of chromatin compaction, stress response, gene expression, and DNA repair. The H4K16ac mark is also enriched during normal aging in the temporal lobes of the human brain, as well as in senescent mammalian cells and aging models in yeast. Interestingly, the H4K16ac mark is reduced in AD. The correlation of H4K16ac levels with normal aging suggests that this modification may protect against neurodegenerative diseases and that changes in its levels may predispose individuals to the development of such diseases, primarily AD[32].

Interestingly, silencing transcription factor RE1 (REST), also known as the neuron restrictive silencer factor, is a specific transcription factor that binds to the RE-1/NRSE response elements and recruits a set of co-repressors to silence the transcription of neural genes. In this respect, REST is associated with HDAC1/2 enzymes, histones methyltransferase such as G9a (methyltransferase of H3K9), as well as CoREST, LSD1,

MeCP2, and C-terminal binding protein, which are related to transcriptional repression. REST is expressed at low levels in differentiated neurons[33]; therefore, REST represses genes that promote cell death and AD, induces stress response genes, and protects neurons against oxidative stress and β-amyloid toxicity. REST induction is a result of normal aging in human cortical and hippocampal neurons, and the involved signaling pathways include the Wnt signaling. However, the expression of REST is reduced in AD. In AD, REST is not detected in the cell nucleus but appears in autophagosomes along with misfolded pathological proteins[34]. Thus, the presence of REST during aging is correlated with preservation and longevity, suggesting that REST may generate neuroprotection in the aging of the brain while its decrease may lead to neurodegeneration[34].

MODELS TO STUDY AD EPIGENETICS

The importance of epigenetic mechanisms in AD has been demonstrated through studies using cell cultures, transgenic animal models for AD, and induction of AD by the injection of β-amyloid 1-42 in rats, as well as antemortem and postmortem studies of samples from AD patients. For example, antemortem and postmortem studies have been performed on monozygotic twins, in which one twin was diagnosed with AD, while the other twin did not present any type of dementia. The twin with AD was a chemical engineer who, due to his work, maintained constant contact with pesticides. This twin developed his first symptom of the disease at 60 years old, characterized by a progressive memory loss, with death at 76 years. His twin brother was also a chemical engineer, with the same education, but in a different work environment; this twin died at the age of 79 years of prostate cancer, but without cognitive damage. The presence or absence of AD was confirmed after death[35]. These studies highlight the importance of epigenetics in AD progression in individuals, even among those with identical genetics.

Although models of transgenic mice are a key component of AD studies, none of these models have been able to produce all characteristics of AD. There is still a need for an "ideal" model that develops all clinical and pathological features, ranging from cognitive and behavioral deficits to molecular aspects, including b-amyloid plaques, tau tangles, synaptic and neuronal loss, and neurodegeneration. Different transgenic mouse models have been generated with several modifications to promote the production and accumulation of β-amyloid and tau protein (Table 1).

EPIGENETIC MECHANISMS OF ALZHEIMER DISEASE

DNA methylation in AD

The methylation and demethylation processes of DNA are altered in AD. In one study, the levels and distribution of 5-mC and 5-hmC were evaluated in several regions of the brain during the aging of wild-type and triple transgenic (3xTG–AD). The researchers observed a global reduction in 5-mC and an increase in 5-hmC in the brain of aged 3xTG–AD mice in comparison with the wild-type[36]. These data suggest an abnormal establishment of permissive chromatin, which may lead to an increase in several markers linked to AD[36] (Figure 1).

Decreases in the DNA methylation of hippocampal and cerebral cortex cells have generally been observed in AD. For instance, in cortical neurons from postmortem AD brains, the 5-mC levels were lower than those of healthy controls. Similarly, low 5-mC levels have been reported in the hippocampus, cerebral cortex, and cerebellum of AD patients[37-39]. Furthermore, some methylation maintenance factors, such as DNA methyltransferase 1 and methylated DNA-binding 2, were found to be decreased in the AD hippocampus in contrast to healthy controls[39]. Interestingly, in the above-mentioned study on monozygotic twins, 5-mC was found in the neurons, microglia, and astrocytes of the healthy twin, but not in the brain of the AD twin, demonstrating a reduction in DNA methylation in several cell types in the AD brain[35].

In contrast, the expression of several genes increases upon reduced DNA methylation (hypomethylation), although many of these reports have not been fully validated. Some examples include the *APP* gene, which encodes the *APP*. *APP* gene is silenced by methylation of its promoter region; however, during aging, this gene is demethylated, promoting its expression and consequently, the accumulation of β-amyloid in the brain[40-43]. Nevertheless, some studies suggest no changes in the DNA

Table 1 Mouse models used for the study of Alzheimer disease

Strain	Promoter used	Proteins expressed	Pathogeny	Ref.
3xTG–AD	Thy1 and mPS1	Mutant of *APP* (hAPP695, Swedish mutation), PS1 (*PSEN*1, M146V) and tau (hTau-4R0N, P301L)	Mice containing these mutations develop β-amyloid plaques and NFTs resembling the brain with AD	[36,60, 96,97]
CK-p25	tetO (tet operator)	These mice overexpress the truncated form of p35, p25	p25 activates CDK5 (cyclin-dependent kinase 5), implicated in AD. CK-p25 mice develop neuronal loss, β-amyloid accumulation and loss of synaptic terminations in the hippocampus and cortex as well as memory deficits	[57,98, 99]
APPPS 1-21 / *HDAC6*$^{-/-}$ crossbred	Thy1	Mutated *APP* (KM670/671NL) and the mutated presenilin 1 (L166P)	Mice develop β-amyloid plaques leading to cerebral amyloidosis, dystrophic synaptic boutons, hyper-phosphorylated tau, inflammatory responses and the impairment of cognitive function	[64, 100, 101]
TgCRND8	Hamster PrP	hAPP695 Swe/Ind	The brain of mice contains plaques formed by depositions of β-amyloid, leading to inflammation and cognitive impairments. There is also neuronal loss, accumulation of NFTs, and neuritic changes similar to those observed in AD	[93, 102, 103]
Tg19959	Hamster PrP	hAPP695 with two familial mutations (Swedish and Indiana mutations: K670N/M671L and V717F, respectively). (FVB X 129S6F1 background)	Mice overexpress β-amyloid 1-42 peptide and Bace1 forming plaques	[5,93, 102, 104]
Tg2576-*APP* swe crossbred	Hamster PrP and Mouse PrP	The Swedish mutation (hAPP695) and m/hAPP695^{3} (extra and intracellular regions of mouse β-amyloid, a human β-amyloid sequence and the Swedish mutations of β-amyloid, K594N/M595L)	These mice develop β-amyloid plaques deposition and memory deficits	[94, 105, 106]
APP/PSI	Thy	Mutated *APP* (KM670/671 NL) and mutated presenilin 1 (L166P)	Mice show dystrophic synaptic, hyperphosphorylation of tau, gliosis, and neuronal loss in the dentate gyrus as well as impairment in reversal learning	[95, 101]

NFT: Neurofibrillary tangles; APP: β-amyloid precursor protein; PSEN: Presenilin; HDAC: Histone deacetyltransferase; 3xTG: Triple transgenic; AD: Alzheimer disease.

methylation status of the *APP* gene for a healthy brain in comparison with AD[44,45]. Moreover, normal brain samples were compared with postmortem AD brain samples, and it was reported that the promoter of the *BRCA1* gene, which encodes a DNA repair protein, is hypomethylated in the AD brain. Under these conditions, this gene has a high expression level, whereas the BRCA1 protein appears to be sequestered by tau aggregates. Thus, alterations in the expression and functions of BRCA1 may be involved in the deterioration of AD[46]. In addition, it has been reported that the hypomethylation of intron 1 of the triggering receptor expressed on myeloid cells 2 gene, which is principally expressed in microglia, may induce inflammation pathways associated with AD development. Importantly, triggering receptor expressed on myeloid cells 2 expression is augmented in the hippocampus and leukocytes of AD patients, suggesting its potential as a biomarker for this disease[47-49].

However, studies have also reported augmented DNA methylation in regulatory regions for some genes involved in AD. For instance, methylation of an alternative promoter for the rare coding variant in the phospholipase D3 gene is increased in the AD hippocampus[50], affecting the function of rare coding variant in the phospholipase D3 protein in the processing of *APP*. Furthermore, increased methylation of the promotor for the brain-derived neurotrophic factor (*BDNF*) gene, which encodes a key protein implicated in the maintenance of adult cortical neurons and cognoscitive functions, has also been reported in the brain and peripheral blood of AD patients[51-53]. In contrast, it has been reported that methylation of the phosphatidylinositol binding clathrin assembly protein gene in blood cells from AD patients is most likely related to disrupted cognitive functions, as the phosphatidylinositol binding clathrin assembly protein is involved in modulating the production, transport, and abundance of β-amyloid peptide[54]. Therefore, the DNA methylation status associated with the expression of several specific genes is altered in AD, occurring in the hippocampus, cerebral cortex, and some in peripheral blood cells, demonstrating their potential as putative biomarkers for this disease.

Histone acetylation and deacetylation in AD

With transgenic mice as an AD model, it has been determined that HDAC2 is

primarily expressed in the hippocampus and prefrontal cortex and reduces the density of dendritic spines, the number of synapses, synaptic plasticity, and memory formation in comparison with wild-type mice[55,56]. Additionally, by studying the neurodegeneration process in the brain of CK-p25 mice, researchers detected high levels of HDAC2 and reduced histone acetylation for genes related to learning and memory, as well as the inhibition of genes related to neuroplasticity[57]. HDAC1/2 expression abated in microglia from AD transgenic mice reduced amyloid load, improving cognitive function[58]. Furthermore, in the study of monozygotic twins (previously mentioned), HDAC2, and HDAC9 expression levels in peripheral blood cells were higher in the AD twin than in the healthy twin[59]. Also, increased HDAC3 expression is associated with a decreased memory in the brains of AD mouse models, whereas the loss of HDAC3 expression, experimentally induced in the dorsal hippocampus, appears to improve memory[60-62] (Figure 2).

In addition, HDAC6 is increased in the cortex and hippocampus of AD patients, and it colocalizes with the tau protein in the hippocampus, whereas a reduction in the levels of tau is observed when HDAC6 levels are decreased[63]. It was reported that reduced HDAC6 levels might improve cognitive activity in double transgenic mice (APPPS 1-21 /HDAC6$^{-/-}$)[64]. In contrast, in one report that used *HDAC*4 knock-out mice suggested that the lack of *HDAC*4 reduces learning and memory. Thus, some HDACs are overexpressed in AD patients, whereas HDAC4 seems to decrease synaptic plasticity and memory formation[65].

The acetylation of tau promotes pathological tau aggregation, but SIRT1 can deacetylate tau. Nonetheless, SIRT1 expression levels are decreased in the cortex in AD[66-68]. Additionally, several AD mouse models have been treated with *HDAC* inhibitors such as sodium butyrate, trichostatin A, and valproic acid and have shown improvements in learning and memory, some of which result from reduced β-amyloid levels[69,70]. Some studies on histone modifications have also been reported. For example, a reduction in H3K18ac and H3K23ac has been identified in the AD brains[71]. Other potentially relevant modifications are H3K27me3 and H3K4me3 modifications, which are enriched in DNA-hypermethylated regions and are associated with aging and AD[72].

Non-coding RNAs

Several non-coding RNAs are implicated in the differentiation, connections, and functions of the neurons, as well as in neurodegenerative processes, participating in proteostatic mechanisms, mitochondrial dysfunction, apoptosis, and neurotrophic factor reduction in the neurons and glial cells[73]. One study reported that intracerebroventricular injection of β-amyloid 1–42 resulted in an AD pattern and deregulation of non-coding RNAs in the hippocampus region[74] that included miRNAs and lncRNAs. Interestingly, it has been proposed a putative role as blood-based biomarkers for some miRNAs in AD[75]. For instance, the expression of several miRNAs such as miRNA-29a, miRNA-29b, and miRNA-29c was reduced with an increase in *BACE1* (β-secretase 1) expression, which is essential for β-amyloid production (Figure 3). This deregulation has been detected in the brain and peripheral blood of AD patients[76-78]. In contrast, increased levels of miRNA-7, miRNA-9-1, miRNA-23a/miRNA-27a, miRNA-34a, miRNA-125b-1, miRNA-146a, and miRNA-155 have been observed in postmortem AD neocortex samples in comparison with healthy controls[79]. Furthermore, increased miRNA-135a and miRNA-384 Levels and decreased miRNA-193b levels have been found in the serum of AD patients compared with healthy controls[80]. Moreover, the upregulation of miRNA-200b and miRNA-200c was detected in Tg2576 transgenic mice. However, the exogenous overexpression of miRNA-200b and miRNA-200c reduced β-amyloid secretion in *in vivo* and *in vitro* experiments[81]. Also, it has also been reported that miRNA-107, miRNA-125b, miRNA-146a, miRNA-181c, miRNA-29b, and miRNA-342 Levels are lower in blood cells from AD patients than in blood from healthy patients[82].

Additionally, miRNA-206 is highly expressed in *APP*/*PSEN*1 transgenic mice, mainly in plasma, CSF, and hippocampal regions, correlating with a downregulation of *BDNF*, and this phenomenon has also been observed in AD patients[83-85]. In another AD model (Tg2576 AD), as well as the brain samples from AD patients, the miRNA-206 expression is also increased and negatively regulates the expression of *BDNF* at the transcriptional level, which affects synaptic plasticity and memory[84,86]. Moreover, miRNA-206 can be detected in early dementia patients through biopsy of olfactory epithelia[83]. AM206, the antagomir of miRNA-206, prevented the pathogenic effect of β-amyloid 1-42 and increased the levels of *BDNF*, synaptic density, and neurogenesis after intranasal administration[86,87]. Hence, miRNA-20 is considered as a reliable biomarker for AD. Another of the more reliable biomarkers that have been proposed is

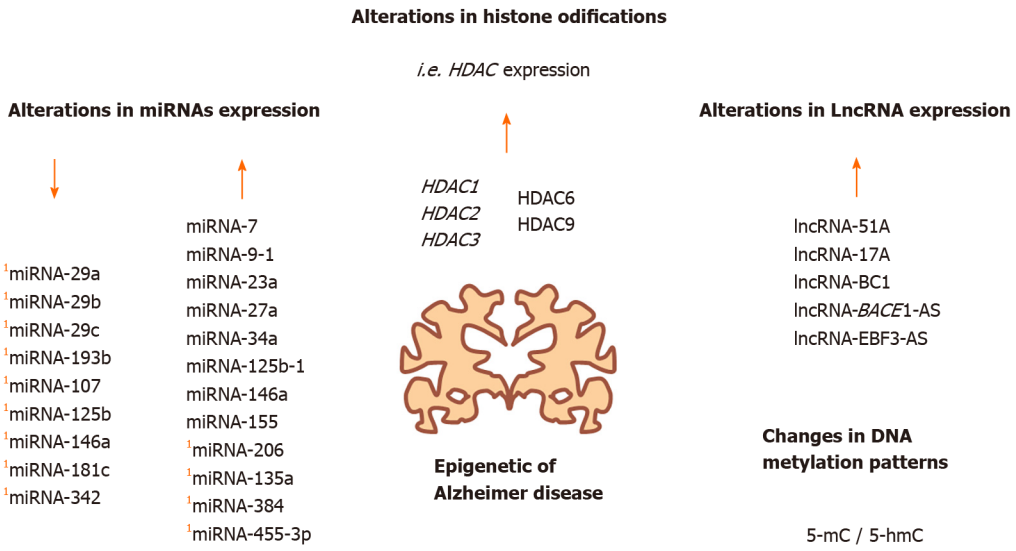

Figure 2 Epigenetics changes in Alzheimer disease. Alterations in epigenetic mark patterns are observed in the brain (primarily the hippocampus, cortex) and peripheral blood ([1]) under Alzheimer disease conditions in comparison to normal conditions. HDAC: Histone deacetyltransferase; DNA: Deoxyribonucleic acid; miRNA: MicroRNA; lncRNA: Long non-coding RNAs; 5-mC: 5-methylcytosine; 5-hmC: 5-hydroxymethylcytosine.

miRNA-455-3p since its expression is upregulated in serum samples and brain tissues from AD patients, and these results are also corroborated in transgenic mice and AD cell lines (skin fibroblasts and lymphoblast cells). Thereby, miRNA-455-3p is also suggested as a potential peripheral biomarker for this disease[88]. Thus, miRNAs appear to be relevant indicators of AD progression, and the detection of these miRNAs in the blood may be a powerful tool for this disease (Figure 3).

Similarly, microarrays and RNA-seq studies have found significant changes in lncRNA expression in the AD brain compared with control brain samples[89,90]. For instance, an increase in lncRNA-51A has been reported in AD; lncRNA-51A is known by modulating the splicing of sortilin-related receptor 1 (an important gene for traffic and recycling of the β-amyloid precursor), reducing the synthesis of sortilin-related receptor 1 variant A. Consequently, APP processing is altered, and β-amyloid production is increased, which promotes AD progression[91]. As another example, lncRNA-17A levels are also increased in AD and regulate the alternative splicing of the $GABA_B$ receptor; moreover, this lncRNA promotes β-amyloid secretion in response to inflammatory signals[92]. A previous study identified an antisense lncRNA for *BACE1*, which was termed lncRNA-*BACE*1-AS. Through *in vivo* and *in vitro* assays, lncRNA-BACE1-AS was shown to confer stability to *BACE1* mRNA, which increases β-amyloid production and AD development. Furthermore, β-amyloid 1-42 overexpression and stressing factors appear to increase lncRNA-BACE1-AS levels, resulting in amyloid protein aggregation. In both AD patients and a murine AD model (APP 695SWE/IND; TgCRND8 or Tg19959), lncRNA-BACE1-AS expression is augmented[93]. Moreover, it has been observed that lncRNA-BC1 is highly expressed in brains from Tg2576-APPswe mice, another mouse model used to study AD. This lncRNA promotes the translation of *APP* mRNA, which increases the production and aggregation of β-amyloid peptide[94]. Levels of lncRNA- early B cell factor 3 (*EBF*3)-AS, *i.e.*, an antisense lncRNA for *EBF*3, are increased in the hippocampus of APP/PSI mouse model for AD. The authors of this study proposed that lncRNA-EBF3-AS may induce EBF3 expression to stimulate neuronal apoptosis under AD conditions[95].

CONCLUSION

Despite the complexity involved in understanding and treating AD, epigenetic mechanisms have emerged as potential elements of this disease. To date, studies have reported several modifications in the epigenome of brain cells under AD conditions respect to normal conditions. Importantly, some of these changes have been detected in peripheral blood cells, rendering these changes as promising biomarkers for this disease. The application of HDAC inhibitors has demonstrated beneficial results for several AD mouse models[96-105], impacting β-amyloid levels, tau phosphorylation, and hippocampus dendritic spine restoration, improving learning and memory. Thus, the

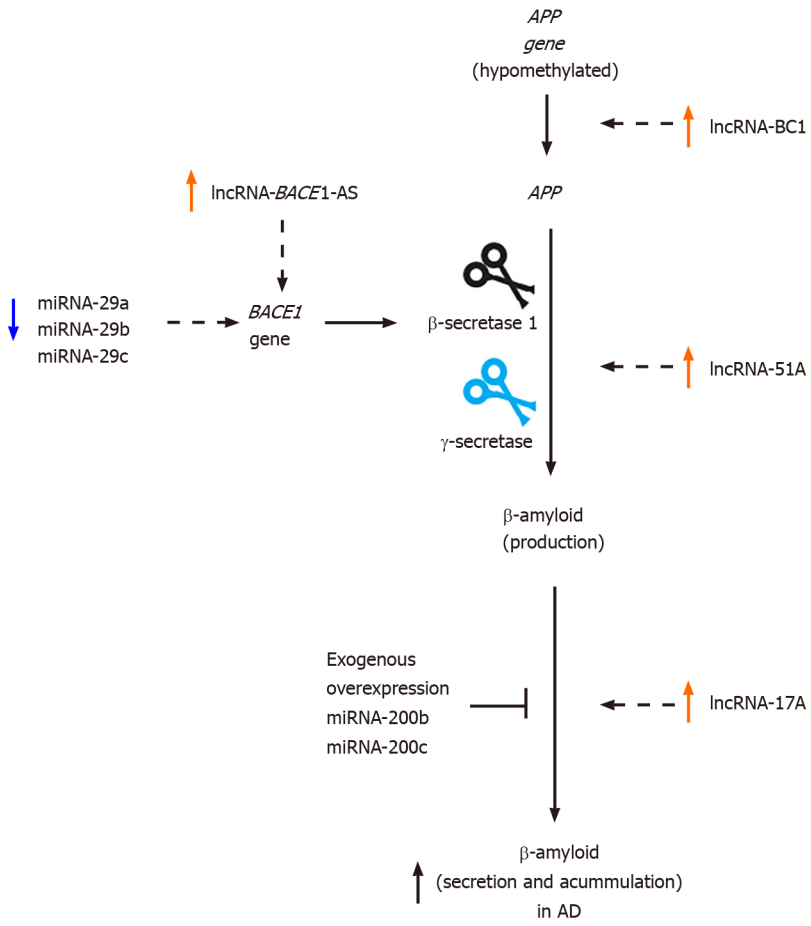

Figure 3 Regulation of β-amyloid production pathway and its epigenetic alterations. The deregulation of some miRNAs and some epigenetic modifications increase the production, secretion, and accumulation of β-amyloid. Furthermore, some long non-coding RNAs (lncRNA), such as lncRNA-51A, lncRNA-BC1, lncRNA-17A and lncRNA-BACE-AS are involved in the overproduction of β-amyloid. Pink and blue arrows indicate an increase and decrease of expression, respectively. AD: Alzheimer disease; APP: β-amyloid precursor protein; lncRNA: Long non-coding RNAs; miRNA: MicroRNA.

modulation of the epigenetic modifications in AD, and the identification of epigenetic determinants for healthy aging and those for pathological neurodegeneration requires to be deeply studied (Figure 4).

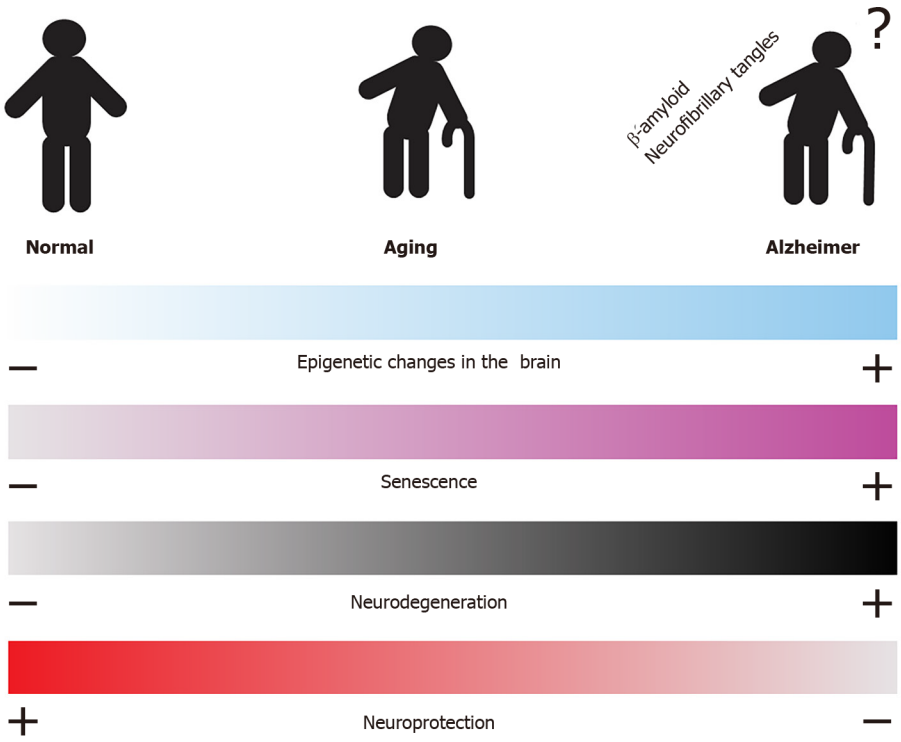

Figure 4 Relevance of epigenetics in aging, neurodegeneration, and Alzheimer disease. Changes in the epigenomes are detected during aging, from young to older people, and in Alzheimer disease (AD) patients. The senescent cells are accumulated during aging. The expression of genes associated with neurodegeneration is increased, whereas the expression of genes associated with neuroprotection is decreased during aging. All these changes are increased in AD patients. A connection between these processes may be critical in aging and during the progression of AD.

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REVIEW

Current understanding of glucose transporter 4 expression and functional mechanisms

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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

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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.

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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

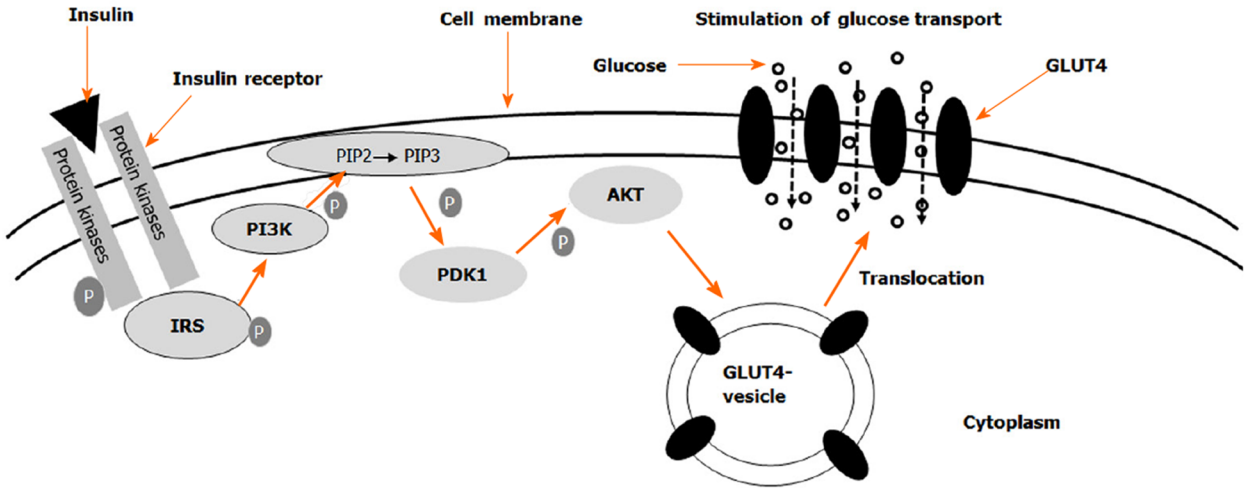

Figure 1 Schematic of insulin-induced translocation of glucose transporter 4 from cytosol to the cell membrane. The binding of insulin to its receptors initiates a signal transduction cascade, which results in the activation of Akt. Akt acts on the glucose transporter 4 (GLUT4) containing vesicles in 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

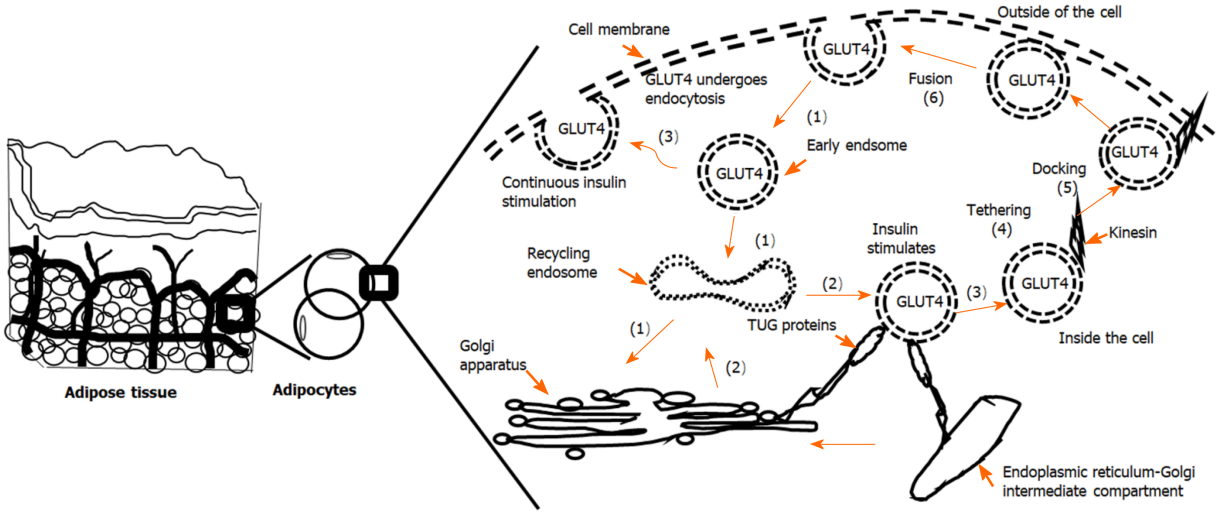

Figure 2 The movement of glucose transporter 4 in adipocytes. Adipose tissue is made of adipocytes. In adipocytes, glucose transporter 4 (GLUT4) can be found in the cell membrane and in the cytosol. The translocation of GLUT4 from cytosolic vesicles to the cell membrane leads to elevated glucose uptake, 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.

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MINIREVIEWS

Deciphering the modifiers for phenotypic variability of X-linked adrenoleukodystrophy

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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

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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.

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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.

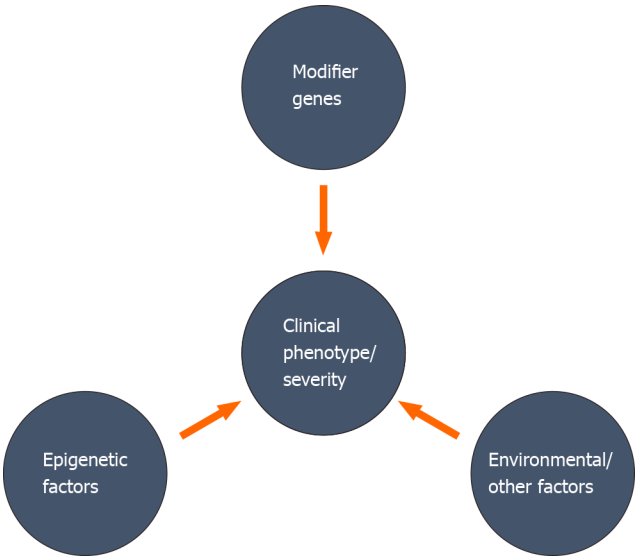

Figure 1 Possible modifiers associated with disease phenotypic variability. Phenotypic heterozygosity observed in a monogenic disorder can be due to the association of modifying factors such as genetic, epigenetic, and/or environmental factors, commonly termed as "modifiers."

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.

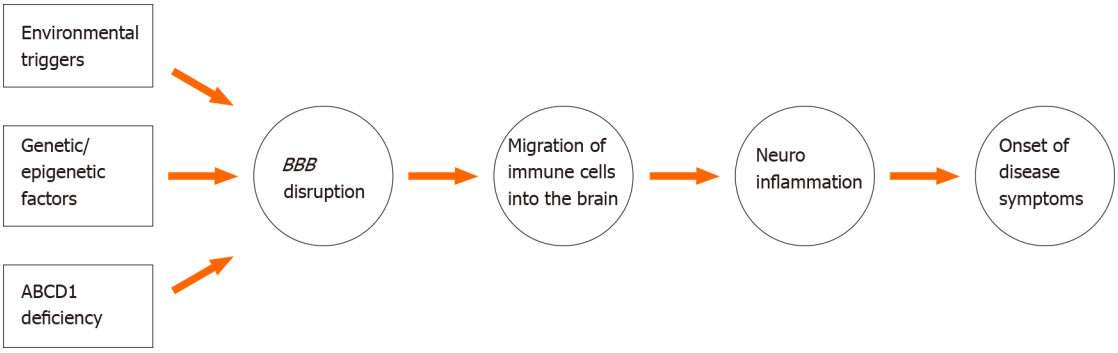

Figure 2 Factors determining the onset of symptoms of cerebral demyelination. Migration of immune cells into the brain could be a rate-limiting step in the appearance of symptoms of cerebral demyelination. Onset of the migration across the blood brain barrier could be precipitated by environmental triggers, 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.

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ORIGINAL ARTICLE

Observational Study

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

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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}

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(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.

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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

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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 (1.5×10^8 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.

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