



Introduction

Otitis media (OM) is a complex disease with various risk factors for its development, chronicity, and recurrence as well as a multifactorial etiology that is yet to be fully understood. An intricate interplay among environmental, genetic, microbial, anatomic, and immunological factors contributes to the pathogenesis of OM. Within this context, young age, lack of breastfeeding, use of pacifiers, day-care attendance or overcrowding at home, and exposure to tobacco smoke and particulate matter, to name a few, have been identified as environmental risk factors for the development of OM [1–3].

Within the otolaryngology practice, the presence of family history remains one of the most important inquiries made during an evaluation of a child with OM. Inherited or genetic factors were found to influence the early onset and development of OM, with a higher incidence observed in certain populations [4–7]. For example, the frequency of OM is increased in children with chromosomal abnormalities such as Down syndrome (MIM 190685) and Turner syndrome (MIM 300082), in which the affected chromosomes often contain numerous genes of various functions [8, 9]. Chromosome 21, which is affected in Down syndrome, contains many genes that are involved in the immune system, particularly those that play a role in interferon signaling, thereby causing a predisposition to viral and bacterial infections [10–15]. Compounded by distinct craniofacial structures of Down syndrome and its associated dysfunctions, such as small middle ear compartments, poor palatal tone, and maldeveloped Eustachian tubes, individuals with Down syndrome often experience OM that does not resolve easily, requiring multiple tympanostomy tube procedures, and sub-

sequent persistent tympanic membrane perforation and hearing loss [16–19]. Turner syndrome is similar in its clinical manifestation with immune alterations found in T cells and immunoglobulins (Igs) along with craniofacial abnormalities of the palate, jaw, and ears that range from the external to inner ear system [20–22]. As a result, affected individuals are predisposed to a range of otologic problems from recurrent or chronic OM to conductive or sensorineural hearing loss and tympanic membrane pathology [9, 23].

The socioeconomic impact of health-care costs and delay in speech and language for OM-affected individuals underline the need for further research in OM. Although our current understanding of the main contributors of genetic susceptibility to OM remains scant compared to our overall genetic knowledge of other common diseases, such as cardiovascular disorders and cancer, continued efforts toward the identification of the determinants of human susceptibility and host genomic responses to OM will help elucidate its multifactorial etiology. Recent advances in genomic databases, sequencing, and analytic techniques will benefit the ongoing genetic investigation into OM and ultimately lead to a better understanding of its pathogenesis, which, in turn, can improve strategies for its prevention and treatment.

Human DNA Studies

Heritability

Heritability, in the narrow sense, is an estimate of the proportion of the variance in phenotypic or trait values under study (in this case, OM), which is due to genetic factors [24]. Because OM is a complex trait, heritability has been estimated using data from related individuals as was done in twin studies performed in three cohorts from Norway, the United States, and the United Kingdom (UK). The initial twin study from Norway in 1997 consisted of 2850 twins, which was then followed up in 2004 with an enlarged cohort of 4247 twins [25, 26]. In the initial study, gender differences

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were detected in female twins who had higher heritability for OM at 74% compared to males with 45% heritability [25]. In the follow-up study that included an additional 1397 twins, gender-based differences for heritability estimates were not observed, although the overall heritability for OM remained high at 61–72% [26].

In Pittsburgh, a prospective cohort study of 168 same-sex twins and 7 same-sex triplets was conducted to determine the heritability of time or duration of middle ear effusion (MEE) [27]. The same cohort was reassessed after 5 years, and it was determined that the heritability of the time with MEE as a phenotype remained consistent [28]. Heritability was 73% in the initial study and 72% in the follow-up study, and both had $p < 0.001$ [27, 28].

Rovers et al. (2002) assessed the heritability of both OM and chronic airway blockage in a prospective study of 1373 twins from the UK [29]. The heritability of acute OM was estimated to be 57%. For both OM and chronic airway blockage, an increasing genetic contribution to the phenotypic variance was observed at ages 2 years (49%), 3 years (66%), and 4 years (71%). However, no gender differences were observed [29].

Valuable information can also be gleaned from familial studies with multiple OM-affected siblings as in the study by Hafrén [30]. Heritability estimates of recurrent acute and chronic OM were determined in their survey of 2436 children, who underwent surgery for OM, and their relatives from 590 Finnish families. Chronic OM had lower heritability (22%) compared to recurrent acute OM (38%), and the overall heritability of all types of OM was estimated at 48% [30]. As expected, heritability estimates are higher in identical twins than in other familial relations due to the greater

similarity in both genetic makeup and environmental background of twins. Nonetheless, these heritability studies, albeit with various heritability estimates, phenotypic definitions, ethnic background, and cohort composition, confirm the significant contribution of genetic factors to OM susceptibility.

Genetic Linkage Studies

Genetic linkage studies test whether a pattern of inheritance of a disease or trait coincides with the genotypes of families: genetic linkage is therefore a test of co-segregation of a variant or variants inherited together (e.g., a haplotype) with the known disease status of family members. The main metric for linkage is the logarithm of odds (LOD) score, which is usually considered statistically significant if a LOD score of 3.3 or greater is obtained for a variant or haplotype within pedigrees that co-segregate a disease or a trait [31]. To obtain a significant LOD score, either a large enough pedigree or a consanguineous family with multiple affected individuals is needed. Alternatively, smaller families that each on its own cannot obtain linkage but, when analyzed together, can lead to mapping of significant loci may be used. Before the advent of next-generation sequencing, genetic variants that are distributed across the genome were genotyped and then tested for linkage in order to identify a genomic region where the pathogenic variant is statistically the most likely to occur. Early linkage studies using different cohorts of families identified multiple loci in various regions of the human genome, most significantly on chromosome 10q (Table 10.1). It should be noted, however, that in many of the mapped loci,

Table 10.1 Genomic loci mapped for otitis media susceptibility in linkage studies using family data

Loci	Studies	LOD score	Significance and candidate genes within the loci
19q13.42–q13.43	Daly et al. [32]	LOD 2.61 ($p = 5.3 \times 10^{-4}$)	Leukocyte receptor cluster (<i>LRC</i>)—cluster of genes with polymorphic functions; mainly prevents immune cell activation [38]
	Chen et al. [35]	LOD 3.75 ($p = 1.6 \times 10^{-5}$)	Zinc finger and zinc finger-related genes (<i>ZNF71</i> , <i>ZNF8</i> , <i>ANF304</i>) Inflammasome protein complex (<i>NLRP13</i> , <i>NLRP5</i> , <i>NLRP8</i>)
10q26.3	Daly et al. [32]	LOD 3.78 ($p = 3.0 \times 10^{-5}$)	<i>ADAM8</i> —identified in allergen-induced asthma, expressed in leukocytes [193, 194]
	Rye et al. [33]	Z_r 2.69 ($p = 3.6 \times 10^{-3}$)	<i>ADAM12</i> —involved in the epidermal growth factor receptor signaling pathway [41, 194] <i>Dedicator of cytokinesis 1 (DOCK1)</i> —phagocytosis of apoptotic cells, skeletal and respiratory muscle tissue development in embryogenesis [42] <i>TCERG1L</i> and <i>PPP2R2D</i> —an intergenic region Transcription elongation regulator-like protein (<i>TCERG1L</i>)—suggested in fasting insulin, attention deficit disorder Protein phosphatase 2A (<i>PPP2R2D</i>)—a modulator of the TGF- β /Activin/Nodal pathway [33, 43]

Table 10.1 (continued)

Loci	Studies	LOD score	Significance and candidate genes within the loci
3p25.3	Daly et al. [32]	Unconditional: LOD 0.60 Conditional: 10q LOD 2.43 19q LOD 1.84	Presumed to be involved in the gene–gene interaction between 19q13.42 and 10q26.3 <i>HRH1</i> —an inflammatory mediator [195] <i>IRAK2</i> —mediates inflammatory gene expression via NF- κ B [196]
17q12	Casselbrant et al. [197]	LOD 2.83 ($p = 7.0 \times 10^{-5}$)	Adaptor-related protein complex2, beta 1 subunit (<i>AP2B1</i>)—CD8 ⁺ downregulation [198] Chemokine C–C motif ligand (<i>CCL</i>)—recruitment of eosinophils [199]
10q22.3	Casselbrant et al. [197]	LOD > 2 ($p = 1.8 \times 10^{-3}$)	<i>SFTPA2</i> —part of surfactant protein A, which regulates the phagocytosis of pathogens; expressed in the Eustachian tube [45]
	Rye et al. [33]	Z_{lr} 1.64 ($p = 0.05$)	–
7q33	Casselbrant et al. [197]	LOD > 2 ($p = 1.0 \times 10^{-3}$)	–
6p25.1	Casselbrant et al. [197]	LOD > 2 ($p = 2.6 \times 10^{-3}$)	–
4p15.2	Casselbrant et al. [197]	LOD > 2 ($p = 3.0 \times 10^{-3}$)	–
15q26.1	Allen et al. [59]	$p = 9.1 \times 10^{-7}$	<i>KIF7</i> —the region is related to the splice site; regulates sonic hedgehog [200] and Indian hedgehog [64] via protein trafficking <i>TICRR</i> —initiation of DNA replication [65]
5p15.33	Allen et al. [59]	$p = 0.045$	Tubulin polymerization-promoting protein (<i>TPPP</i>)—located at the intron; affects microtubule function [63]
2q31.1	Allen et al. [59]	$p = 1.3 \times 10^{-5}$	Non-coding region: possible role in the regulation of LDLR (ch19), which is expressed in ciliated airway epithelial cells [201]

the pathogenic variants responsible for OM susceptibility remain unknown. This is partly due to the lack of genetic studies performed in families with OM and potentially due to genetic heterogeneity for OM where families carry variants within multiple genes that are mostly rare or private variants, such that current family cohorts lack the power to detect significant OM loci. Below is a review of loci that have been mapped using families with OM.

19q13.42–q13.43

Daly et al. (2004) first identified the 19q13.42–q13.43 region as a suggestive susceptibility locus (LOD 2.61, $p = 5.3 \times 10^{-4}$) using genotype data from Minnesotan families with OM [32]. Approximately 100 annotated genes are present in this region, but many of their functions remain unknown. The leukocyte receptor cluster (*LRC*) genes were suggested as potential candidate genes within the 10q locus. Within this region are genes encoding the leukocyte Ig-like receptor (*LIR*; also known as Ig-like transcripts) and the killer cell immunoglobulin-like receptor (*KIR*), which are transmembrane proteins expressed on cells of immune function for various protein activation or inhibition [33]. Such proteins include tyrosine phosphatases Src homology region 2 domain-containing phosphatase (SHP)-1 and/or SHP-2 [34]. This region was replicated by Chen et al. (2011), who fine-mapped the locus to chromosome 19q13.43 using additional single nucleotide variant genotypes (LOD 3.75,

$p = 1.6 \times 10^{-5}$) [35]. The additional candidate genes identified within the 19q locus include genes related to zinc fingers, the tumor necrosis factor-alpha (TNF α), bone morphogenetic protein (BMP), and fibroblast growth factor-beta (FGF β) pathways, lymphocyte activation, and the inflammasome protein complex, all of which regulate innate immunity in response to harmful stimuli [35–37].

10q26.3

In an initial linkage study for OM using the Minnesota family cohort, the 10q26.3 locus was identified with a LOD score of 3.78 ($p = 3.0 \times 10^{-5}$) and was replicated in another cohort of Western Australia-based trios by Rye et al. in 2014 (Z_{lr} 2.69, $p = 3.6 \times 10^{-3}$) [32, 33]. Herein, trios are composed of the OM-affected individual (proband) and both parents, without affected or unaffected siblings. Within the 10q26.3 locus, the Minnesotan and Australian studies identified candidate genes encoding a disintegrin and metalloproteinase (ADAM) domain. ADAM8 is found in leukocytes in response to allergen exposure in asthma and is also upregulated in epithelial cells of the airway during allergic inflammation [38, 39]. The function of ADAM12 is most strongly associated with cell adhesion and fusion, extracellular matrix restructuring, and cell signaling [40]. It has been found to be upregulated in the middle ear in response to tobacco smoke [41]. Other genes implicated in this region include *DOCK1* (MIM 601403), which is involved in phagocytosis; *TCERGIL*

encoding transcription elongation regulator-like protein, and *PPP2R2D* (MIM 613992), which modulates the modulator of the transforming growth factor (TGF)/Activin/Nodal pathway [33, 42, 43].

10q22.3

Chromosomal region 10q22.3 has also been identified (LOD >2, $p = 1.81 \times 10^{-3}$) and replicated with suggestive evidence (Z_{lr} 1.64, $p = 0.05$), though not reaching statistical significance [2, 33]. *SFTPA1* (MIM 178630) and *SFTPA2* (MIM 178642) are genes within this region that together encode the human surfactant protein (SP-A) and have previously been implicated in OM [44]. SP-A haplotype and genotype variations have been observed in children with the first episode of OM before 6 months, and common variants in these genes were associated with protection against OM in infants at risk of asthma [45, 46]. SP-A is expressed in the middle ear mucosa and in the Eustachian tube and is known to contribute to innate immune responses by increasing the phagocytosis of otopathogens [45–48].

Candidate Gene Association Studies

Using a gene of interest, candidate gene association studies estimate the frequency with which the minor allele, or the less prevalent allele in the background population, is found in OM-affected patients in comparison to a control group. Healthy or unaffected individuals that are related to the OM-affected

patients within the same families, or unrelated healthy individuals with no previous history of OM, may be used as controls (case–control study). Numerous studies have been conducted, particularly using candidate genes, relating to inflammation and innate immune responses, but many candidate gene studies will not lead to a significant association when a more stringent threshold for genome-wide significance is applied. Nonetheless, some genes that were initially identified in smaller case–control association studies, such as the *HLA* and *ABO* (MIM 110300) genes, were also deemed significant loci in genome-wide association studies (GWASs) for OM [49–56].

Genome-Wide Association Studies (GWASs)

A GWAS is currently the starting point in identifying the loci of interest. It assesses the association of variants spread throughout the genome with the trait or disease under investigation and, in contrast to candidate gene association studies, does not assume an association between a locus of interest and the trait. Instead, a GWAS employs an agnostic approach to identifying the loci of interest, which may be a regulatory variant often found in the non-coding region, a haplotype encompassing variants that are inherited together, or a gene harboring multiple rare variants associated with the trait. For OM, a series of GWASs have been conducted using common variant genotypes from microarrays, with each describing independent findings (Table 10.2). With advances

Table 10.2 Otitis media susceptibility genes identified from genome-wide association studies

Study	SNP	Cases	Controls	Cohort	Significant associations ^a	Candidate genes
Rye et al. 2012 [33]	2,524,817	416	1075	Western Australian Pregnancy Cohort (Raine) Study	rs6755194 ($p = 8.3 \times 10^{-7}$) rs1862981 ($p = 2.2 \times 10^{-5}$)	<i>CAPN14</i> <i>GALNT14</i> <i>BPIFA</i> gene cluster
Allen et al. 2013 [59]	324,748	373	229	University of Minnesota University of Pittsburgh [24]	rs1110060 ($p = 9.1 \times 10^{-7}$) rs10497394 ($p = 1.5 \times 10^{-8}$) rs10775247 ($p = 6.3 \times 10^{-5}$)	<i>KIF7</i> <i>TICRR</i> <i>TPPP</i>
Einarsdottir et al. 2016 [66]	964,193	829	2118	Finnish	rs16974263 ($p = 1.8 \times 10^{-7}$) rs268662 ($p = 1.6 \times 10^{-6}$) rs4150992 ($p = 3.4 \times 10^{-6}$)	<i>PLD3</i> <i>SERTAD1</i> <i>SERTAD3</i> <i>HIPK4</i> <i>PRX</i> <i>BLVRB</i>
van Ingen et al. 2016 [70]	460,000	825	7936	European-descent Americans	rs2932989 ($p = 4.4 \times 10^{-8}$) rs3767498 ($p = 1.25 \times 10^{-6}$) rs12725646 ($p = 4.3 \times 10^{-5}$) rs255142 ($p = 1.9 \times 10^{-6}$) rs9514552 ($p = 2.1 \times 10^{-5}$) rs12888576 ($p = 7.8 \times 10^{-6}$) rs2809139 ($p = 3.2 \times 10^{-6}$) rs8036951 ($p = 3.8 \times 10^{-5}$) rs10409140 ($p = 4.4 \times 10^{-5}$)	<i>FNDC1</i> <i>KIF21B</i> <i>CACNA1S</i> <i>ASCL5</i> Intergenic region near <i>MIR205HG</i> <i>CRHR2</i> <i>INMT</i> <i>ARGLU1</i> <i>BDKRB2</i> Intergenic region near <i>C14orf177</i> <i>FAM189A1</i> <i>TPM4</i>

Table 10.2 (continued)

Study	SNP	Cases	Controls	Cohort	Significant associations ^a	Candidate genes
Pickrell et al. 2016 [49]; Tian et al. 2017 [53]	560,000–950,000	46,936	74,874	European-descent Americans	rs681343 ($p = 3.5 \times 10^{-30}$) rs1978060 ($p = 1.2 \times 10^{-19}$) rs2808290 ($p = 5.1 \times 10^{-16}$) rs7174062 ($p = 3.5 \times 10^{-14}$) rs4329147 ($p = 9.6 \times 10^{-12}$) rs8176643 ($p = 3.7 \times 10^{-11}$) rs1802575 ($p = 1.5 \times 10^{-10}$) rs5829676 ($p = 1.8 \times 10^{-10}$) rs72931768 ($p = 2.6 \times 10^{-9}$) rs35213789 ($p = 3.8 \times 10^{-9}$) rs114947103 ($p = 5.4 \times 10^{-9}$) rs13281988 ($p = 9.8 \times 10^{-9}$) rs67035515 ($p = 1.6 \times 10^{-8}$) rs73015965 ($p = 3.8 \times 10^{-8}$)	HLA genes Several intergenic regions <i>FUT2</i> <i>ABO</i> <i>TBX1</i> <i>MKX</i> <i>AUTS2</i> <i>CDHR3</i> <i>PLG</i>

^a *p*-values in bold font indicate those that passed the genome-wide significant thresholds of the study

in computational efficiency, continuous decline in the cost of sequencing, and increasing availability of large-scale biobank data with genotypes and OM phenotypes, the discovery of additional novel findings from GWASs, which include both common and rare variants, is possible in the near future.

In the first GWAS on OM, Rye et al. (2012) used data from Australian trios and identified three novel candidate genes/gene clusters, namely, (1) rs6755194 within chromosome 2p23.1, which is either upstream of or intronic to two isoforms of *CAPN14* (MIM 610229); (2) rs1862981, also located on 2p23.1, intronic to *GALNT14* (MIM 60822) and proximal to *CAPN14*; and (3) *BPIFA* clusters, including *BPIFA1* (MIM 607412), *BPIFA2*, and *BPIFA3* on the genomic region 20q11.21 [57]. However, these findings could not be replicated in an independent cohort [58]. Other genes identified in this study with trends toward association were linked to the TGF- β pathway [57].

In the Minnesota-based cohort, Allen et al. (2013) identified an intergenic locus rs10497394 between the genes *CDCA7* (MIM 609937) and *SP3* (MIM 601804) on 2q31.1, which was subsequently replicated in an independent Pittsburgh-based US cohort as being associated with both chronic OM with effusion and recurrent acute OM [59]. This locus was found to regulate the expression of *LDLR* (MIM 606945) on chromosome 19, a gene that is expressed in ciliated epithelial cells of the airway with its transcribed protein predicted to be a binding site for human rhinovirus C, a known pathogen for upper respiratory tract infections and OM [60]. Newer data from the Genotype-Tissue Expression (GTEx) database showed that the rs10497394 variant significantly regulates RNA levels of *CDCA7* in thyroid and arterial tissues; however, GTEx does not include data from middle ear tissues [61]. *CDCA7* mutations cause immunodeficiency, centromeric instability, and facial anomalies syndrome (MIM 616910), wherein hypogammaglobulinemia leads to recurrent life-threatening

infections [62]. Additional loci on chromosomes 5 and 15 (rs386057, rs1110060, and rs10775247) were also identified but did not reach genome-wide significance in the replication study [59]. The genes affected by these three variants are involved in microtubule function, regulation of mammalian sonic hedgehog and Indian hedgehog pathways, and DNA replication [63–65].

Einarsdottir et al. (2016) found three variants, all on chromosome 19, to be associated with childhood OM in the Finnish population [66]. None of the identified regions overlapped with those previously found in association with OM on chromosome 19. Genome-wide significance was established for rs16974263, a variant intronic to the *PRX* (MIM 605725) gene, which was found in association with chronic OM with effusion in a UK cohort, albeit with opposite directions of effect [66]. Out of the several genes identified within this region are three candidate genes, *PLD3* (MIM 615698), *SERTAD1* (MIM 617850), and *BLVRB* (MIM 600941), which are previously known to be associated with immune function with expression found in macrophages [67, 68]. In the GTEx database, the rs16974263 variant regulates either the RNA levels or splicing of isoforms for *SERTAD3*, *HIPK4* (MIM 611712), *PLD3*, and *PRX* in various tissues [61]. In particular, *SERTAD3* is expressed in the mucosal tissue and its protein inhibits the replication of influenza A virus upon induction by type I interferon responses during an infection [61, 69].

Additional studies in Americans of European-descent identified genome-wide significance for a variant on chromosome 6 (rs2932989) that alters the methylation status of the gene encoding fibronectin type III domain containing 1 (*FNDC1*, MIM 609991) [70]. Although its function is not clearly elucidated, the study found an upregulation of this gene in the middle ear tissue under pro-inflammatory conditions [70]. Other candidate genes from suggestive loci largely contained those related to immune responses.

Finally, 14 genome-wide significant loci were identified by Pickrell et al. (2016) and Tian et al. (2017) using GWAS data from 23&Me [49, 53]. Among these loci, the OM-associated genes included *FUT2* (MIM 182100) and *ABO*, both involved in glycosphingolipid biosynthesis, as well as *TBX1* (MIM 602054) and *MKX* (MIM 601332), which encode transcription factors. Genes important for embryogenesis and neurodevelopment (*FGF3* (MIM 164950) and *AUTS2* (MIM 607270)), as well as those implicated in asthma (*CDHR3* (MIM 615610)), and spontaneous chronic OM in mice (*PLG* (MIM 173350)) were also found in association with OM [71–73]. To date, only 4 of these 14 loci, *FUT2*, *TBX1*, *ABO*, and *CDHR3*, have been replicated in independent human cohorts with additional evidence from functional or multi-omics studies performed for *FUT2* and *CDHR3* [49–52, 74–78].

To identify pathways that are potentially important for OM susceptibility, 21 genes with $p < 5 \times 10^{-5}$ available in the literature were selected from candidate gene association studies and GWAS results. Using NetworkAnalyst software [79–81], these genes were then used as an input for defining protein–protein interaction networks. Within these networks exist subnetworks that are involved in significant pathways, highlighting the underlying pathophysiology and serving as potential targets for novel therapeutics (Fig. 10.1). The major pathways identified included a rhythmic process, a viral pro-

cess, negative regulation of an apoptotic process, transcription, transcription by RNA polymerase II, chromatin remodeling, regulation of cell cycle, and circadian rhythm (Table 10.3). These identified pathways suggest that basic

Table 10.3 Significant pathways identified from network analysis of genes implicated in otitis media from exome sequencing and genome-wide association studies

Significant pathways	<i>p</i> -value	Genes
Rhythmic process	7.9×10^{-17}	<i>PPARG</i> <i>NCOA2</i> <i>CREBBP</i> <i>TP53</i> <i>PRKDC</i> <i>UBE3A</i> <i>PML</i> <i>EP300</i> <i>NRIP1</i> <i>PPARGC1A</i> <i>SP1</i> <i>MAGED1</i> <i>TOP1</i> <i>CREB1</i> <i>DDX5</i> <i>SFPQ</i> <i>KDM5A</i> <i>GNB2L1</i> <i>NFKB2</i> <i>HNRNPU</i> <i>MLL</i> <i>HDAC1</i> <i>HDAC2</i> <i>THRAP3</i>
Viral process	1.99×10^{-16}	<i>MDM2</i> <i>YWHAE</i> <i>COPS6</i> <i>KAT5</i> <i>CREBBP</i> <i>TP53</i> <i>STAT3</i> <i>CD4</i> <i>TSG101</i> <i>NEDD4L</i> <i>UBE3A</i> <i>PML</i> <i>HNRNPA1</i> <i>EP300</i> <i>FYN</i> <i>SP1</i> <i>STAT1</i> <i>IRAK1</i> <i>RELA</i> <i>MAP3K7</i> <i>MMP1</i> <i>LCK</i> <i>RB1</i> <i>HSPD1</i> <i>TOP1</i> <i>SRC</i> <i>CREB1</i> <i>TBP</i> <i>YWHAB</i> <i>CEBPA</i> <i>RAN</i> <i>RBX1</i> <i>SUMO1</i> <i>GNB2L1</i> <i>SET</i> <i>PAK2</i> <i>TRIM28</i> <i>CUL1</i> <i>CUL2</i> <i>EIF4A2</i> <i>DAXX</i> <i>IKBKG</i>
Negative regulation of the apoptotic process	2.95×10^{-13}	<i>ROR2</i> <i>MDM2</i> <i>AKT1</i> <i>EGFR</i> <i>YWHAZ</i> <i>PTK2B</i> <i>STAT5B</i> <i>IL6</i> <i>TMF1</i> <i>TP53</i> <i>RAF1</i> <i>PRKDC</i> <i>CD38</i> <i>STAT3</i> <i>SMAD3</i> <i>NFKB1</i> <i>MED1</i> <i>IRAK1</i> <i>RELA</i> <i>IL10</i> <i>UBC</i> <i>COPS5</i> <i>ADA</i> <i>WNT1</i> <i>JUN</i> <i>HSPA1A</i> <i>HSP90AB1</i> <i>HSPD1</i> <i>SRC</i> <i>MIF</i> <i>CBL</i> <i>DUSP1</i> <i>AKT2</i> <i>MSX2</i> <i>SNCA</i> <i>CDKN1A</i> <i>BCL6</i> <i>PPIID</i> <i>PAK2</i> <i>HDAC1</i> <i>NTRK2</i> <i>HDAC2</i> <i>BAG1</i>

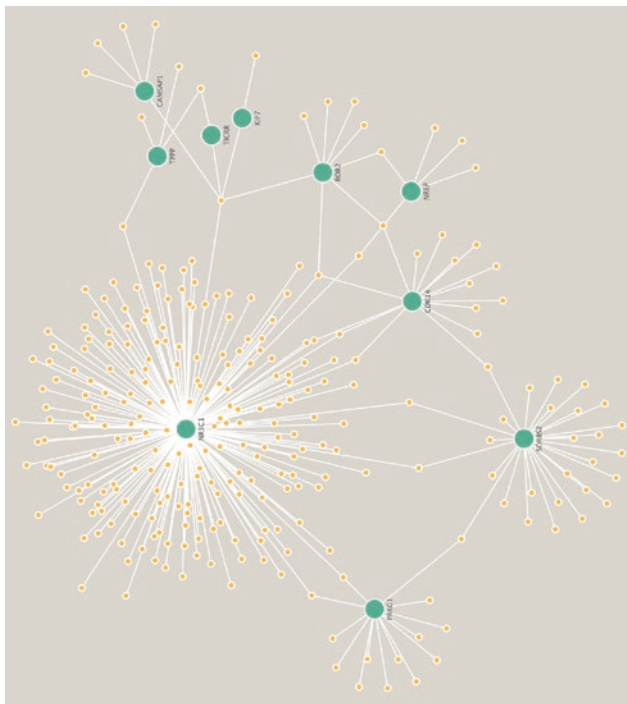


Fig. 10.1 Protein–protein interactions in the network analysis of genes implicated in otitis media from exome sequencing and genome-wide association studies. The green nodes represent proteins encoded by input or seed genes with a $p < 5 \times 10^{-5}$ from exome sequencing and genome-wide association studies. The orange nodes represent proteins that are found in association with the seed proteins within a significant cellular process

Table 10.3 (continued)

Significant pathways	<i>p</i> -value	Genes	
Transcription, DNA-templated	6.0×10^{-13}	<i>NR3C1</i> <i>PPARG</i> <i>NCOA1</i> <i>NR3C2</i> <i>IRF1</i> <i>IRF8</i> <i>POU1F1</i> <i>NR4A1</i> <i>NR1H3</i> <i>TCF3</i> <i>POLR1D</i> <i>IRF2</i> <i>IRF5</i>	<i>RELA</i> <i>GTF2I</i> <i>JUN</i> <i>POU2F2</i> <i>POU2F1</i> <i>RXRβ</i> <i>CEBPA</i> <i>PPP5C</i> <i>NR1H2</i> <i>NR1H3</i> <i>ASXL1</i> <i>PHF3</i> <i>ARHGAP35</i>
Transcription by RNA polymerase II	6.9×10^{-11}	<i>NR3C1</i> <i>STAT5B</i> <i>TMF1</i> <i>TADA2A</i> <i>ONECUT1</i> <i>TP53</i> <i>TGFB11</i> <i>IRF1</i> <i>STAT3</i> <i>POU1F1</i> <i>MAFF</i> <i>TAF6</i> <i>TRIM24</i>	<i>NR4A1</i> <i>NR1H3</i> <i>SMAD3</i> <i>EP300</i> <i>IRF2</i> <i>NCOR1</i> <i>NFKB1</i> <i>CEBPB</i> <i>PBX1</i> <i>IRF5</i> <i>NFATC2</i> <i>NFATC1</i> <i>GTF2I</i>
Chromatin remodeling	5.96×10^{-9}	<i>TADA2A</i> <i>SMARCE1</i> <i>SMARCD1</i> <i>HMGB1</i> <i>SMARCC1</i> <i>SMARCA4</i> <i>HMGB2</i>	<i>SMARCA2</i> <i>RB1</i> <i>MYB</i> <i>TOP1</i> <i>HDAC1</i> <i>HDAC2</i> <i>DAXX</i>
Regulation of cell cycle	1.3×10^{-8}	<i>CDK11A</i> <i>IRF1</i> <i>STAT3</i> <i>EP300</i> <i>SRSF5</i> <i>MED1</i> <i>CDK14</i> <i>COP5</i> <i>USP16</i>	<i>DTL</i> <i>ABL1</i> <i>JUN</i> <i>RB1</i> <i>SFPQ</i> <i>CCND3</i> <i>SFN</i> <i>GNB2L1</i>
Circadian rhythm	4.6×10^{-8}	<i>EGFR</i> <i>NCOA2</i> <i>EP300</i> <i>NR1P1</i> <i>PPARGC1A</i> <i>NCOR1</i> <i>HNRNPR</i>	<i>JUN</i> <i>TOP1</i> <i>CREB1</i> <i>HDAC1</i> <i>NTRK2</i> <i>THRAP3</i>

cellular processes are involved in OM susceptibility with viral infection as a primary target for OM prevention and treatment.

Exome Sequencing

The coding regions or exome, i.e., all the exons comprising the parts of all genes that are transcribed and translated into proteins, only make up approximately 1% of the human

genome. With growing knowledge and advancements in technology, exome sequencing allows for the assessment of variants within all coding regions of the genome and has become a cheaper alternative for identifying pathogenic variants for various diseases, particularly for rare variants with strong phenotypic effects. Using exome sequence data, rare pathogenic variants in three genes, *A2ML1* (MIM 610627), *FUT2*, and *SPINK5* (MIM 605010), were found in association with OM in an Indigenous Filipino pedigree and has since been replicated in various OM cohorts of different ethnicities [74, 82–84].

The *A2ML1* gene encodes a protease inhibitor that is specifically expressed in the middle ear [83]. It is similar to alpha-2-macroglobulin (A2M), also a protease found in the middle ear that is associated with recurrent acute OM [85]. Two loss-of-function *A2ML1* variants, i.e., a frameshift variant and a splice variant, were identified in a cohort of Indigenous Filipino population with an estimated 50% prevalence of OM [83]. The same frameshift variant in *A2ML1* was identified in US-based otitis-prone children with early-onset severe OM ($p = 3.34 \times 10^{-14}$) [83]. An additional 25 variants of the same gene were found in probands with OM from different cohorts [82, 83]. Moreover, differential expression analysis of RNA sequence data from Coloradan children with OM revealed co-upregulation of *A2ML1* with genes (e.g., *SPINK5*) that are involved in several pathways, including keratinocyte differentiation [82]. Follow-up microbiota studies showed a significantly higher relative abundance of the *Leptotrichia* spp. in the middle ears of individuals with *A2ML1* variants [84, 86]. In contrast, for a rare pathogenic *SPINK5* variant that was also identified in the Indigenous Filipino population through exome sequencing and linkage analysis, a greater biodiversity in the oral cavity and increased relative abundance of *Microbacteriaceae* were observed [84]. Notably, *A2ML1* is expressed in the middle ear mucosa, whereas *SPINK5* is faintly localized to the mucosal tissues but strongly localized to the outer ear and tympanic membrane in mouse middle ears [83, 84].

FUT2, located on chromosome 19, encodes 2-fucosyltransferase and is involved in the synthesis of blood group H antigens and the regulation of its expression on various mucosal surfaces [87]. Several common and rare variants within *FUT2* were associated with an increased risk for OM in US trios and various ethnic cohorts [74]. These include two stop variants, p.Trp154* and p.Arg202*, and two missense variants, p.Ala104Val and p.Arg138Cys [74]. For these four *FUT2* variants, levels of A antigen, a common binding spot for potential otopathogens and commensal bacteria, were reduced on the cell surface of mutant epithelial cells [74]. *FUT2* variants also affect gene transcription and alter the mucosal microbiome in the setting of OM. Differential expression analysis revealed downregulation of *FNI*, *KMT2D*, *MUC16*, and *NBPF20* and upregulation

tion of *MTAP* in individuals with the *FUT2* p.Trp154* variant [75]. Changes in regulation of these four genes were also seen in the middle ears of mice inoculated with the human otopathogen non-typeable *Haemophilus influenzae* (NTHi), except upregulation of *Fnl* found in the inoculated mice [75]. Additionally, the *FUT2* variant was associated with an increased load of otopathogens within the middle ear with concordant decrease in relative taxa abundance of commensal bacteria in the nasopharynx [75].

Also utilizing exome sequencing, Jamieson et al. reported two genes, *NR3C1* (MIM 138040) and *NREP* (MIM 607332), as candidates for severe OM in a cohort from the Australian Aboriginal population [88]. Although these genes failed to attain genome-wide significance, *NR3C1* and *NREP* implicate a role of gene–environment interaction in the expression of inflammatory modulators due to environmental stress and shifts in the microbiome, respectively [89–93].

Mouse DNA Studies

A critical limitation to human studies is the inability to adequately control for genetic or environmental factors due to variability and heterogeneity across human subjects. Animal models present an alternative method in the exploration of the genetics of OM through the control of genetic and environmental factors and have led to a growing number of genes identified in association with OM [94]. Despite the availability of numerous animal models in the study of OM, the murine model has been largely favored in genetic studies. In all, 99% of mouse genes are homologous to human genes and, consequently, mice and humans share similar physiology and development of many basic functions [95]. Owing to the early mapping of the mouse genome, tools to manipulate mouse genes for phenotyping and susceptibility studies are widely available, along with a growing number of knockout and transgenic mouse models [96].

Gene-driven models and phenotype-driven *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis methods have been used to understand the genes involved in OM in mice. In the gene-driven method, a growing availability of transgenic and knockout mouse strains through efforts such as the Knockout Mouse Phenotyping Program (KOMP2) is harnessed to assess the effect of mutations on OM susceptibility [97]. In the phenotype-driven model, ENU is used as a mutagen to induce random nucleotide changes [95]. This is then followed by screening of the phenotype of interest (e.g., increased OM prevalence), allowing for the discovery of novel genes. Genetically altered mice from either method may express the desired phenotypes, for example, by developing spontaneous OM, or in other studies, changes in OM

expression after inoculation of otopathogens through transbullar injection or pressure-induced translocation of microbes to the middle ear from the nasopharynx [98, 99]. The genes identified thus far mainly involve craniofacial development or innate immune responses. Here, we will discuss a number of genes with a distinctive expression of OM phenotypes, illustrating the key points.

Mice with Craniofacial Abnormalities

Mice with impaired craniofacial development from DNA modifications often develop chronic OM with effusion and are frequently associated with congenital syndromes. Gene-driven models have led to the implication of numerous genes in knockout mice with similar phenotypic observations. *E2f4* is a key transcription factor that interacts with pRB during cell cycle progression [100]. Many *E2f4*^{-/-} mice die early on, but the surviving mice develop OM due to craniofacial defects that increase their susceptibility to opportunistic infections [101]. In humans, mutations in the *EYA* gene family, comprised of nuclear phosphatases that act as transcriptional coactivators, are known to cause branchiootorenal syndrome 1 (MIM 113650) from variants in *EYA1* (MIM 601653) and sensorineural hearing loss (MIM 601316) from variants in *EYA4* (MIM 603550) [102–106]. In mice, deficiency of *Eya4* caused middle ear and Eustachian tube abnormalities, ultimately leading to OM with effusion [107]. Similar findings were observed in haploinsufficient *Ets1* and *Fli1* mice that developed OM and had craniofacial abnormalities consistent with those of the small middle ear cavity, short nasal bone, and malformation of the nasal bony-cartilaginous junction [108]. Specific to the middle ear, anatomical abnormalities, including ossicular fusion to the middle ear wall and stapedia malformations, were observed. The genes *ETS1* (MIM 164720) and *FLI1* (MIM 193067) lie within a genomic region of the human genome on the long arm of chromosome 11, which is deleted in Jacobsen syndrome (MIM 147791); Jacobsen syndrome includes craniofacial dysmorphisms and isoimmune thrombocytopenia among its clinical features [109]. These genes are E26 transformation-specific (ETS) transcription factors that are expressed in neural crest cells during embryogenesis [110–112].

ENU-derived *edison* and *Jeff* variants both lead to spontaneous chronic OM and mild craniofacial defects in mice [113, 114]. In both *edison* and *Jeff* variants, their respective mutations in the mouse genes *Nisch* and *Fbxo11* are also associated with faulty innate immune responses (further described below) [115]. *Eya4* is another gene with a possible regulatory role in the immune system [116].

Studies of Innate Immune Responses in Mice

An innate immune response is broad and encompasses cellular functions of macrophages and neutrophils to mucosal integrity and ciliary function. As such, the genetic findings related to innate immunity are accordingly diverse. Toll-like receptors (TLRs) are a class of pattern recognition receptors involved in innate immunity at the cellular level. Numerous *Tlr* mutations have been identified through knockout mouse models (gene-driven) as causing or prolonging otitis media in mice. *Tlr4* deletion caused a delay in immune responses, leading to an early development of OM, which was often chronic [117, 118]. Knockout of *Tlr2* and *Tlr9* also resulted in similar findings with prolonged or severe infections [119, 120]. A similar phenotype was observed with the deletion of *Myd88*, which encodes an adaptor protein recruited in response to Tlr and interleukin (IL)-1 for activation of downstream signaling pathways [121, 122]. After *Myd88* knockout, a delay in macrophage and neutrophil recruitment resulted in chronic OM and prolonged mucosal thickening, which were more severe than those found with *Tlr* mutations [123].

In response to TLR activation, the NF- κ B pathway is triggered downstream to initiate an inflammatory response [124]. Through phenotype-driven models, a number of genes have been associated with the NF- κ B pathway and OM. As described above, the *edison* mouse variant displayed disruption in immune response in addition to the observed craniofacial abnormalities. The deletion of the *Nisch* gene further impacted the downstream signaling of the NF- κ B pathway as well as the LIMK1 pathway, which is associated with vascular permeability and perturbation that lead to middle ear effusion and mucoperiosteal inflammation [113, 125, 126]. Another ENU-derived mouse strain is *Junbo*, which has a deletion of the *Evi1* gene and demonstrated spontaneous acute OM and chronic OM [127]. *Evi1* encodes a transcription factor that is known to be involved in multiple pathways [128, 129]. In the NF- κ B pathway, *Evi1* binds to a subunit of NF- κ B, preventing its interaction with DNA, thereby down-regulating inflammation [130].

In the TGF- β pathway, the activation of TGF- β causes a cell signaling cascade with SMAD proteins, leading to transcription of target genes, RNA processing, translation of messenger RNA (mRNA), and protein regulation for numerous cellular processes, including craniofacial development and inflammation [131–134]. The genes implicated in association with the TGF- β pathway include *Tgif1*, *Fbxo11*, and *Evi1*. As previously mentioned, the phenotypic changes observed in *Jeff* mice were attributed to a mutation in the *Fbxo11* gene, which encodes for a ubiquitination protein involved in tumor suppression [135, 136]. *Jeff* mice dis-

played nuclear accumulation of the Smad2 protein, which mediates transcription of target genes within the TGF- β pathway [137]. *Evi1*, defects in which are responsible for the OM observed in *Junbo* mice, interacts with Smad3 proteins, resulting in the suppression of the growth-inhibiting mechanism of the TGF- β pathway [127, 129, 138]. Based on the mutagenesis studies that implicated the TGF- β pathway in OM in these gene-based mouse models, TGIF1, a homeodomain protein that acts as a negative regulator of the TGF- β pathway, binds to Smad2 to recruit a co-repressor and subsequently recruits histone deacetylases, leading to inhibition of transcription [132, 139, 140]. In *Tgif1*-knockout mice, conductive hearing loss was associated with chronic effusion, middle ear mucosal thickening, and an increase in goblet cells [141].

Other pathways and functions presumed to be associated with OM include the c-Jun N-terminal kinase (JNK) pathway, mucociliary clearance, mitogen-activated protein kinase (MAPK) pathway, and bony development [76, 129, 142–145]. Despite the vast expansion of genetic knowledge gained from these mouse studies, there are a number of limitations that prevent rapid application of knowledge from mouse models to application in the management of OM in humans. Because the otopathogens in humans differ from those in mice, these mouse models require inoculation of human otopathogens into the middle ear, which often cause a shorter course of OM in mice. There are also immunological and anatomical differences. The lymphocyte-rich immune system of mice versus the neutrophil-rich immune system in humans as well as the lack of mastoid air cells in mice require additional consideration [146]. At the genetic level, alternative splicing and innumerable factors contributing to complex disease processes like OM require additional complementary studies in humans.

RNA Studies

There are various types of RNAs, each serving numerous functions beyond protein translation to the regulation of gene expression. Examination of RNA profiles can thus provide a more accurate and detailed picture of the cellular mechanisms at play for various disease processes. A messenger RNA (mRNA) is a single-stranded RNA, complementary to the DNA, used as codes for protein synthesis. In contrast, a microRNA (miR) provides insights into gene expression. A microRNA is a 23 nucleotide-long RNA that functions to control gene expression posttranscriptionally by binding to the 3' untranslated region (UTR) of a target mRNA [147]. Accordingly, investigators have studied mRNAs and miRNAs in order to understand OM pathophysiology.

mRNAs

mRNA expression in OM has been studied through the assessment of single gene transcription or whole transcriptome of various cells within the middle ear. Single-gene studies performed on human samples showed differential expression of aquaporins, C-type lectin receptors, mucins, beta-defensins, and cytokines in different types of OM, implicating the roles of cellular homeostatic and inflammatory responses in OM pathogenesis [148–152]. Differential expression of the protooncogene C/EBP-homologous protein (*DDIT3*, MIM 126337) was observed in otitis-prone children, associating the frequency of OM with the endoplasmic reticulum stress response through the PERK (protein kinase RNA-like endoplasmic reticulum kinase) signaling pathway [153].

Various methods of genetic expression analysis, including reverse transcription PCR (RT-PCR), microarray, and RNA sequencing (RNA-seq), have been used to profile the genetic expression of middle ear epithelial cells (MEECs) in association with OM. The types of information that can be gathered from these studies are wide-ranging. Inoculation of murine models with a common human otopathogen, NTHi, helped identify differential expression of 3657 genes, most of which are involved in innate immune response modulation, cell marker variation, and recruitment of neutrophils and macrophages [154]. The substantial role that innate immunity plays in OM was reinforced in the study by Ryan et al. (2020) with single-cell transcriptome analysis in mice [155]. Considerable cellular diversity in the mouse middle ear mucosa was observed with identification of 17 distinct cell types, all with expression of innate immune genes [155]. The cellular diversity of the middle ear mucosal tissue is further demonstrated in the variable types of MEECs identified [156, 157]. In the process of MEEC differentiation, approximately 500 genes that are associated with secretory proteins and ciliogenesis were upregulated [158]. Such findings provide a pathway for improving the characterization of the unrestrained responses of MEECs with the cellular remodeling that occurs in OM. Stabenau et al. (2021) studied human MEECs in OM with effusion in comparison with normal mucosal cells and identified 1282 differentially expressed genes [159]. The functions of identified genes encompassed inflammation, bacterial immunity, mucociliary clearance, cellular proliferation and transformation, and auditory cell differentiation. Of these functions, the most upregulated genes involved mucin production, immunity, and cell cycle regulation.

MicroRNAs (miRs)

miRs have diverse actions and targets that are context-specific. Most commonly, miRs bind to the 3'UTR of an

mRNA in perfect pairs or with imperfect complementation but with additional base pairings at the 5' of the mRNA [160]. miRs have also been observed to bind to 5'UTRs, the coding region of RNA, or directly to gene promoters [161–163]. The complexity in understanding the mechanism of miRs is also due to the ability of one miR to bind to numerous distinct mRNAs. Furthermore, one mRNA can be bound by different miRs, with each pairing leading to a distinct outcome [161]. As a result of miR binding to mRNA and 3'UTR, translational suppression can occur as deadenylases and decapping factors are recruited [164]. With its protective ends exposed, the resulting mRNA strand will be prone to degradation. Binding of miR to 5'UTR and the coding region causes silencing of expression, but its interaction with a promoter region has been observed to lead to transcription [165, 166]. The presence of miRs in various cellular compartments, the abundance of miRs, mRNAs, and their various combinations, and the miRNA-induced silencing complex mediating translational inhibition make the function of miRs dynamic [162].

The role of miRs in OM is increasingly being examined. In vitro analysis of human MEECs revealed differential expression of 15 genes as a result of both up- and downregulating functions of miRs. These changes ultimately led to an increase in cell differentiation, endocytosis, cellular communication, $\text{I}\kappa\text{B}/\text{NF-}\kappa\text{B}$ cascade, developmental process, complement activation, innate immune response, and cell adhesion [167]. miR-146 has been implicated in numerous inflammatory diseases owing to its negative regulatory role in activating TLRs and its ability to fine-tune signaling cascades. In OM, miR-146 expression increased in response to in vitro exposure to pro-inflammatory cytokines, which, in turn, was correlated with middle ear mucosal thickness and observed decline in the expression of tumor necrosis factor receptor (TNFR)-associated factor 6 (TRAF6), a modulator in the TLR pathway [168].

Exosomal release of endocytic vesicles leads to extracellular release of mRNAs, miRs, and proteins to be then transported between cells [169, 170]. Particularly, exosomal miRs have been found to influence expressions of distant cells and promote positive or negative effects on pro-inflammatory signaling of the receiving cell [171–174]. Human MEECs produce a baseline level of extracellular miR, the microRNAome, which is composed of 110 different miRs during in vitro stimulation [175]. For example, stimulation by NTHi induces elevation in the levels of five distinct miRs, namely, miR-378a-3p + miR-378i, miR-200a-3p, miR-378g, miR30d-5p, and miR-222-3p, all known to target genes related to innate immunity [175–180]. The targeted mRNAs are involved in apoptosis, cancer, cell growth, and the IL-8 pathway mediated by CXCR1/2 that activates NF- κ B, oxidative stress, inflammation, chemotaxis, angiogenesis, and neutrophil functions [175]. Of the miRs, miR-320e has been

further associated with the presence of allergies in children with OM [181]. In an exosomal miR analysis in patients with middle ear effusion, 17 miRs unique to middle ear effusions compared to serum controls were identified, with the most abundant being miR-223-3p [170]. These exosomal miRs regulate a total of 442 target genes, most of which were involved in the IL-8 signaling process and were also mediated by CXCR1/2. miR-223 regulates innate immune response, protease activity, and a few key functions of neutrophils [182, 183].

Another form of RNA that plays a role similar to miR is long non-coding RNA (lncRNA). As the name suggests, it is longer than miR with its length greater than 200 nucleotides and does not code any proteins [184, 185]. It has the ability to interact with DNA, RNA, and proteins and modulate transcription, epigenetic changes, RNA and protein stabilization, translation, and posttranslational modifications [186–189]. Interestingly, it can also interact with miR to modulate cellular function. In OM, the lncRNA nuclear-enriched abundant transcript 1 (NEAT1) targets miR-495 to activate p38 MAPK, allowing the release of inflammatory cytokines and promoting the expression of genes involved in acute inflammatory responses [190–192].

The extent of miR interactions and their functions are still in the process of discovery with much to uncover. Understanding the role of RNA in the expression of final protein products helps delineate complex and dynamic disease processes such as OM.

Future Directions

Although prevention and diagnostic and treatment strategies are available for OM patients, we still lack the understanding of the progression of OM from acute to chronic forms. This lack of knowledge undermines efforts to develop novel therapies and fine-tune the current management protocols, particularly for patients with undiscovered genetic susceptibility to OM. To reduce the massive burden on global healthcare due to OM, ongoing investigations furthering our understanding of the genetic component of OM susceptibility are important to ultimately deliver effective therapeutic solutions to patients. We must continue to identify OM-pathogenic variants within different populations, especially to include underrepresented groups across the world. Advancements in technology bring about efficiency and speed in our ability to sequence genetic materials, which must be harnessed in studying complex diseases with multifactorial contributors at play such as OM. Currently, many human studies involve patients of European descent. A few available studies on minority groups focus on populations with an especially high incidence of OM; however, it is equally as important to study underrepresented groups without such context. Using

bioinformatics, we can glean the genetic networks at play in various types of OM. The influence of pathogens on gene expression requires an in-depth understanding of the microbiome and gene expression responses to different pathogens. Genetic variations from splicing, epigenetic modifications, and different factors that influence production of proteins are especially important. It is essential to continue harnessing the availability of animal models, especially in studying therapeutic solutions, but even more important is the application of the findings from animal models to treating OM in humans.

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