UNIVERSITÀ DI FOGGIA

PhD in

"Experimental and Regenerative Medicine"

XXIX cicle

"Study of molecular basis of Oculo-Auricolo-Vertebral-Spectrum"

Tutor: Prof. Angelo Vescovi

PhD Student: Francesca Piceci Sparascio

Co-Advisor:Dott.ssa Valentina Guida

Final Dissertation Academic Year 2015-2016

Index

Abstract

Oculo auriculo vertebral spectrum (OAVS; OMIM 164210) is a clinically and genetically heterogeneous disorder originating from an abnormal development of the first and second branchial arches. Main clinical characteristics include defects of the aural, oral, mandibular, and vertebral development. Anomalies of the cardiac, pulmonary, renal, skeletal, and central nervous systems have also been described. The aetiology of OAVS remains largely obscure. Nevertheless, the identification of several families with proof of both autosomal dominant and autosomal recessive inheritance, as well as detection of several chromosomal aberrations, strongly suggest that OAVS has a genetic basis. The advent of next-generation sequencing (NGS) surmounts these issues and changes the landscape of rare-genetic-disease research, with the possibility of identifying genetic disease causative genes at an accelerating time-rate. The aim of this work is to study the OAVS molecular bases identifying genes included in microriarrangements using SNP array technology and new candidate genes throughout targeted or exome sequencing. In a large court of 84 clinically well-characterized OAVS patients, some microduplications and microdeletions have been identified, involving genes belonging to the EYA-SIX- and PAX pathways, previously associated with OAVS. Starting from the genes involved in these microriarrangements, a panel of 78 genes has been drawn, including genes implicated in the molecular pattern of the first and second branchial arch development and genes associated with pathologies with a clinical phenotype overlapping with OAVS. In detail, NGS analysis has identified two missense variants in *GCS* and *PLCB4* genes in two sporadic patients belonging to two unrelated families. Animal models have shown that both of these genes are involved in the early stages of embryonic development and, specifically, in the formation of branchial arches, confirming their role in the etiology of OAVS. Two missense variants and a maternally inherited in-frame deletion were also identified in the *MYT1* gene recently associated with OAVS. *MYT1* gene is involved in the retinoic acid pathway as one of the teratogenic agents associated with OAVS. The identification of these three variants confirms the involvement of *MYT1* gene in the etiopathogenesis of this pathology. A second nonsense mutation in the *HOXA2* gene was also identified in a family only presenting isolated microtia. Retrospective studies on

the only other patient reported in literature, showed that both patients displayed a particular shape of the outer helix of the ear, compared to other isolated microtia forms. Finally, the analysis of the whole exome was performed in four affected subjects, belonging to a family of three generations, in which the clinical features segregated as autosomical dominant way with incomplete penetrance and variable expression. Two splicing variants and a missense variant were identified in the *RNF213*, *SHPRH* and *ITGB4* genes, respectively, in all affected and non-affected members of the family. Unfortunately, none of these genes seems to suggest a possible correlation with the aetiological hypotheses brought up until now. This work, therefore, contributed to the expansion of the microdeletion and microduplication spectrum associated with OAVS, presented the first time a point mutation in the *GCS* gene previously associated with OAVS throughout linkage studies, and the identification of a mechanism of allelic heterogeneity linked to the presence of a mutation in *PLCB4* gene, previously associated with Auricolar Condylar Syndrome, in a patient with a Goldenhar phenotype. Finally, it has been contributed to the identification of the second nonsense mutation in the *HOXA2* gene in a patient with isolated microtia and a distinctive ear morphology. All these findings provide further evidence of the molecular mechanisms underlying OAVS, confirming the genetic heterogeneity of this pathology and helping to identify new genotype-phenotypic correlations. Identification of new candidate genes better provide the basis for appropriate genetic testing, clinical management, genetic counseling, with improvements in risk assessment, prognosis and prevention, and for the development of new therapeutic approaches.

KEYWORDS: Oculo auriculo vertebral spectrum; SNP-array analysis; Next Generation Sequencing

1.Introduction

1.1 Definition

Oculo-auriculo-vertebral spectrum (OAVS) is a rare congenital disorder involving the first and second branchial arch derivatives (that affects primarily aural, oral and mandibular development). The estimated incidence is 1/5,600 (Gorlin., 1990), although a recent work showed an incidence of 1/26370 live birth (Barisic et al., 2014).

This spectrum includes conditions previously known as hemifacial microsomia and Goldenhar syndrome (Goldenhar., 1952), and is clinically heterogeneous, ranging from isolated unilateral microtia to multiple visceral malformation.

Most patients present with unilateral hemifacial microsomia. In addition, epibulbar dermoids and upper eyelid colobomas are found frequently, often associated with macrostomia or facial clefts (Rollnick et al., 1987). Moreover, the involvement of the structures of the face, cardiac, renal, pulmonary and central nervous system anomalies are described.

The first patients with OAVS was described by Professor M. Goldenhar in 1952 as a combination of anomalies such as epibulbar dermoid, preauricular tags, ear malformations and abnormalities of the cervical vertebrae; this condition was clinically defined as Goldenhar syndrome (Goldenhar., 1952). A few years later, in 1963, it was suggested the use of the term oculo-auriculo-vertebral dysplasia to describe a syndrome characterized by epibulbar dermoid and/or lipodermoid, ears and vertebral anomalies (Gorlin et al., 1963). In 1976, finally, Gorlin and Pindborg concluded that the hemifacial microsomia, Goldenhar syndrome and oculo-auriculo-vertebral dysplasia, despite they had different clinical severity phenotype, show clinical features almost overlapping (Gorlin et al., 1976). Only later, in 1989, the research group of Cohen coined the term oculo-auriculo-vertebral spectrum (OAVS), today still used (Gorlin et al., 2010 ; Hartsfield., 2007).

Due to the variable expressivity, there is no consensus regarding the minimum diagnostic criteria for OAVS. After the initial suggestion to consider as a minimum diagnostic criterion the presence of microtia (Gorlin et al., 2010), there are different classification systems for OAVS (Cousley., 1993 ; Rollnick et al., 1987 ; Vento et al., 1991). Those described by Cousley (Cousley., 1993)

and Vento et al. (Vento et al., 1991) focus only on the facial anomalies and do not include other frequently associated anomalies as vertebral anomalies, congenital heart defects or limb anomalies. Rollnick et al. (Rollnick et al., 1987) do not take the laterality of involvement into consideration. More recently, Tasse et al.suggest new clinical diagnostic criteria based on the presence of facial asymmetry, in association with ear anomalies and/or preauricular appendages or fistulas. More in detail, Tasse et al. classifyied 53 OAVS patients into three groups based on the presence of the main clinical findings, consisting of microtia (group 1), microtia/preauricular tags plus hemifacial microsomia (group 2), microtia/preauricular tags plus hemifacial microsomia plus vertebral anomalies (group 3), and further differentiated each group into two subgroups: '*u*' for unilateral and '*b*' for bilaterally affected. (Tasse et al., 2005). This classification takes into account not only the presence of all the clinical findings but also the affected side (monolaterally or bilaterally).

By comparing unilaterally and bilaterally affected patients, it has been observed that patients with bilateral involvement require more surgical and supportive treatment, e.g. physiotherapy and speech therapy suggesting to examine these patients more carefully to detect additional clinical findings. The statistically significant correlation between subgroup and number of additional clinical findings suggests that this new classification might be a useful predictor of the likelihood that associated malformations are present in a patient with OAVS.

Recent evidences in literature suggest to take in consideration isolated hemifacial microsomia when associated with a family history of OAVS as a diagnostic criteria.

1.2 The OAVS clinical phenotype

The main clinical features of OAVS include craniofacial abnormalities that are associated with vertebral, cardiac, pulmonary, renal and central nervous system anomalies. The OAVS patients may also have a low birth weight and postnatal growth retardation. About 60% of patients present with unilateral involvement, while 10-33% of patients show abnormalities in both sides with a more serious involvement of the right side than the left (Tasse et al., 2005). The

hemifacial microsomia is present in 65% of patients (Figure 1A), associated with a medical case of different gravity, which also depends on the presence of mandibular or maxillary hypoplasia. Often the asymmetry is not appreciable in the first years of life, but it starts to become clear only later, around 4 years of a child's life (Gorlin et al., 2010). About 35% of patients with agenesis of the mandibular branch also have microsomia more frequently associated with epibulbar dermoid.

The ear abnormalities may relate to the external pinna, in the form of microtia (Figure 1B), present in 90% of patients, and atresia / stenosis of external ear occurring in 25-50% of cases. The latter anomaly is related to the problem of conductive hearing loss which can be also sensorineural affecting approximately 50% of patients (Barisic et al., 2014).

There can also be present preauricular skin tags or fistula, described in approximately 40-60% of patients, which sometimes can be located at the base of the neck (Figure 1C). The skin tags are the only feature of the syndrome related to epibulbar dermoid because of, histologically they are both choristomas (Gorlin et al., 2010).

Vertebral defects (Figure 1D) are reported in 18-60% of patients with OAVS and include the presence of hemivertebrae in the lumbar, thoracic and cervical spine, or scoliosis, spina bifida and butterfly vertebrae (Barisic et al., 2014 ; Anderson et al., 2005). Radial anomalies were observed in 10% of patients, and mainly concern the hypoplasia or aplasia of the radius and/or the thumb (Gorlin et al., 2010).

They may also be present different types of ocular abnormalities. In about 10% -20% of patients can also be present epibulbar dermoid often associated with clinically more severe Goldenhar phenotype (Figure 1E). Patients with epibulbar dermoid frequently show anomalies both extraocular and palpebral of the tear drainage, microcornea, ptosis, and anophthalmia (Figure 1F). OAVS patients may also present microphthalmia (5-35%), coloboma (5-10%) and epicanthus (10%). In particular coloboma in the upper part of the eyelid is found in 20% of cases , meanwhile aniridia or absence of the lens and the retina is present with a frequency <1% (Barisic et al., 2014 ; Gorlin et al., 2010). In addition to skeletal malformations, the OAVS patients may have congenital heart disease in 5-58% of cases. Typically , in literature are described ventricular septal defects and tetralogy of Fallot with or without the right aortic

arch which alone represented 65% of all cardiac abnormalities noticed (Digilio et al., 2009 ; Gorlin et al., 2010). Finally, even patients with transposition of the great arteries have been described, tubular hypoplasia of the aortic arch associated with a mild coarctation of the aorta, cardiomegaly, PDA (Patent Ductus Arteriosus), pulmonary stenosis, dextrocardia, double outlet right ventricle, aortic arch anomalies, hypoplasia of the external carotid arteries and situs ambiguus (Gorlin et al., 2010).

In 5% of patients was observed the presence of tracheoesophageal fistulas while lung abnormalities ranging from incomplete lobulation until hypoplasia and agenesis, unilateral and bilateral. Generally the absence of the lung appears to be in the same side where the craniofacial anomalies are present.

Urogenital defects are present in the 2.5% -50% of patients, including renal ectopia and mono or bilateral renal agenesis, cryptorchidism and multi cystic kidney (Barisic et al., 2014 ; Ritchey et al., 1994) and also, double ureter, renal artery blood flow abnormalities, hydronephrosis, hydroureter. The presence of imperforate anus are also described. Brain abnormalities in OAVS patients have been repeatedly reported and encompass the presence of tumor (e.g., teratoid rhabdoid tumor, medulloblastoma) (Lafay-Cousin et al., 2009), anterior or occipital encephalocele (Kerckoff Villanueva et al., 2008 ; Gustavson and Chen., 1985 ; Aleksic et al., 1984), obstructive hydrocephalus (Kerckoff Villanueva et al., 2008 ; Aleksic et al., 1984 ; Kumar et al., 2000), pons and brainstem abnormalities (Pane et al., 2004 ; Chong et al.,2015), cerebellar hamartoma (Arzimanoglou et al., 1999), hemispheric hypotrophy (Jena and Duggal., 2006), brain calcifications, midline lipomas (Jeanty et al., 1991 ; Beltinger et al., 1988 ; Thommen et al.,1986), and corpus callosum abnormalities (Aleksic et al., 1984).

Few cohort studies investigated systematically the presence of brain abnormalities by means of MRI (Strömland et al., 2007 ; Rosa et al., 2010 ; Tasse et al., 2005) and found them in a high proportion of OAVS patients (17 – 50%). A recent study (Brotto et al., 2017) validated previous findings, both in terms of brain abnormality high frequency (56% in our cohort) and variability. In addition, Brotto et al.,2017 confirmed a previous observation (Rosa et al., 2010) that brain abnormalities are more frequent among OAVS patients with concomitant ophthalmologic abnormalities, probably because the eye is an outpouching of the primitive brain and likely shares some of its development

milestones. Nonetheless, it has also been found an association with spine or cranial nerve abnormalities that became even stronger when both ophthalmologic and spine abnormalities were present. Brain abnormalities seem to be associated with a more severe phenotype thus suggesting a greater or earlier damage during fetal life.

According to Brotto et al., findings, the detection of eye and spine abnormalities in the context of OAVS should prompt for proper neuroimaging to investigate the presence of brain abnormalities (Brotto et al., 2017).

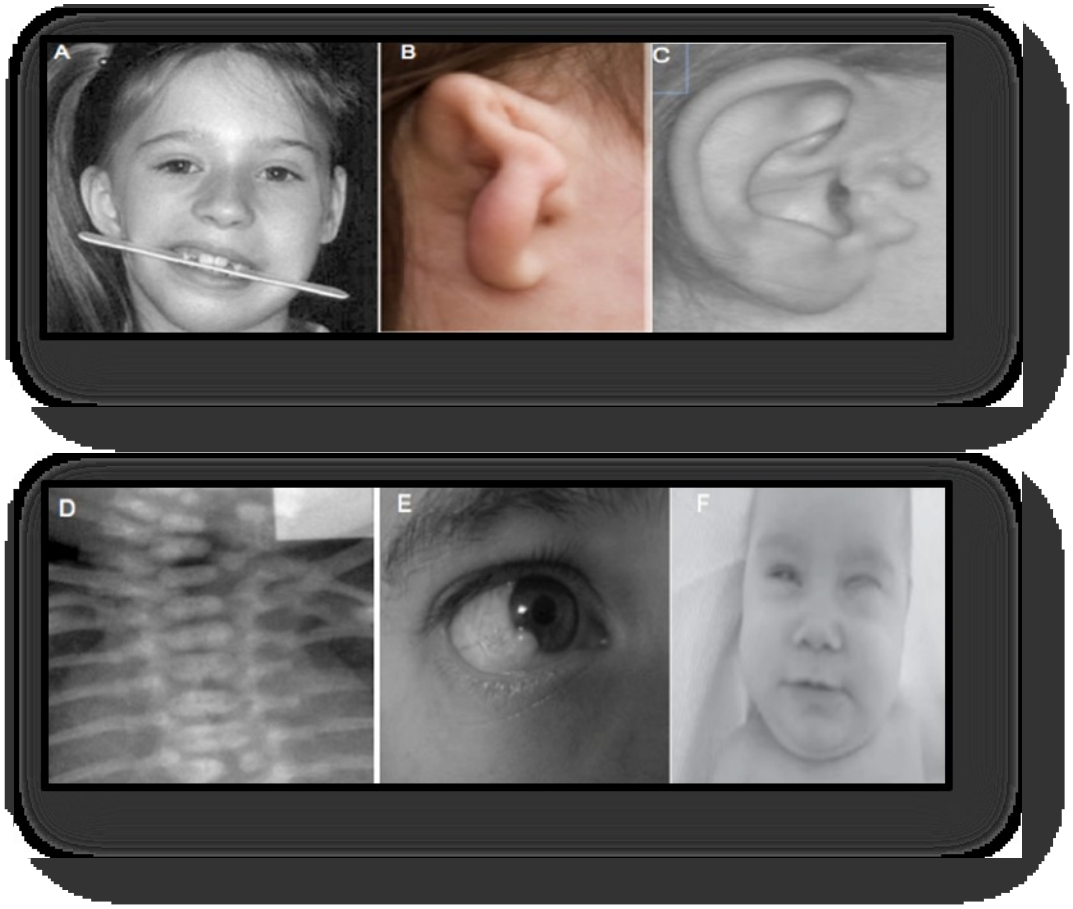


Figure 1. Clinical features OAVs: facial asymmetry (A), microtia (B), preauricular skin tags (C), vertebral anomalies (fusion C6 / C7, T1) (D), epibulbar dermoid (E), microphthalmia (F) (Kelberman et al., 2001 ; Cox et al., 2014 ; Tasse et al., 2007 , Anderson et al., 2005)

1.3 Etiology

Although knowledge of the genetic basis of human disease and its effect on embryonic development has greatly expanded in recent years, the etiology of OAVS are still largely unknown, and the involvement of both genetic and environmental factors have been suggested (Hennekam et al., 2010 ; Heike et al., 2009). Fetal exposure in the first weeks of pregnancy to vasoactive drugs or teratogenic substances, such as pirimidone, thalidomide and retinoic acid , may cause OAVS. In this regard, one of the hypotheses widely shared by the scientific community is that these teratogenic substances lead to an aberrant vascular development in utero. In particular, in a study of 154 pregnancies in which pregnant women were exposed to a retinoid substance (isotretinoin) prescribed for severe cystic acne, not only they were observed a high number of abortions but also the presence, in 21 infants,of cranio-facial malformations, (microtia / anotia, micrognathia, cleft palate), or cardiac malformations (conotruncal heart defects, aortic arch anomalies), and defects of the central nervous system (Lammer et al., 1985).

The OAVS phenotype has also been noticed in infants born to diabetic mothers (Grix., 1982 ; Ewart-Toland et al., 2000 ; Wang et al., 2002). The teratogenic effect of maternal diabetes was confirmed by a study of 21 infants of diabetic mothers who had hemifacial microsomia (67%), microtia (52%), sensorineural hearing loss / conductive (43%), epibulbar dermoid and vertebral fusions (24%) (Ewart-Toland et al., 2000). A plausible explanation is that the state of hyperglycemia in pregnant women alter the migration of fetal neural crest cells. This concept is further strengthened by the observations carried out on mice and chicken embryos, in which the induction of a state of hyperglycemia clearly leads to the presence of defects in cardiac development and neurulation (Wang, et al., 2002). Embryonic hematoma formation or ectodermal non disjunction in early development, which is reminiscent of the occult spinal dysraphisms, have been also suggested to be involved in the pathogenesis of OAVS (Poswillo., 1975 ; Hartsfield., 2007).

Experiments conducted on mouse model by Poswillo., have shown that by injecting intraperitoneally the triazene (antifolate drug) and inducing the formation of bleeding stapedial artery, the murine fetuses showed hypoplasia involving one or both sides of the skull (Poswillo., 1974). Moreover the presence of discordant monozygotic twins and the high frequency of OAVS

children born from assisted reproduction pregnancies suggest that the base of OAVS, may be due to aberrant epigenetic mechanisms. Studies with model organisms reveal how the identity and patterning of vertebrate-specific portions of the skull are epigenetically regulated and how epigenetic dysregulation in cranial neural crest cells induces severe skull vault defects (Haberland et al., 2009). Epigenetic factors have been suggested as a possible pathogenic mechanism leading to histone acetylation-dependent imbalance of allelic expression of the *BAPX1* gene, a member of the NKX family of homeobox-containing proteins, which play a role in skeletal development and patterning of the middle ear, in five patients with OAVS (Fischer et al.,2006). However there are many evidences of the presence of the genetic contribution to the etiology of OAVS, including the presence of family cases. Despite of most OAVS patients are sporadic, familial studies reported that 20-45% of individuals with OAVS has family history (Rollnick and Kaye., 1983 ; Llano-Rivas et al., 1999 ; Klockars et al., 2007) . Rollnick et al (Rollnick and Kaye., 1983) in a study of 97 cases observed that 45% patients had a family history of some features of the disorder, with first-degree relatives being most frequently affected, often with only mild phenotypic expression (for example, isolated preauricular pits or tags), thus suggesting that what is often presumed to be a sporadic event in a family may be the more severe manifestation of a familial condition. The authors have reported that first degree relatives were most often affected (35/433, 8%). Of 176 siblings cited, 11 (6%) were considered affected. Furthermore, the authors observed a broad phenotypic spectrum within families, an aspect that has also been reported by others (Rollnick.,1988 ; Rollnick et al., 1987) and have proposed that familial inheritance is more frequent than originally reported (Rollnick and Kaye., 1983). Subsequently, a genetic predisposition has been proposed based on growing evidence from the literature (Kaye et al., 1992 ; Tasse et al., 2007 ; Vendramini-Pittoli and Kokitsu-Nakata ., 2009 ; Tsai FJ and Tsai CH.,1993 ; Goodin et al., 2009). Both autosomal dominant and recessive family were described. Kaye et al., provided evidence for an autosomal dominant model of inheritance with reduced penetrance troughout a study of segregation analysis performed in 311 members of the families of 74 probands with OAVS (Kaye et al., 1992). Tasse et al., describe an autosomal dominant family where the disease was present with rare bilateral extracranial anomalies (Tasse et al., 2007)(Figure 2). Some authors

have suggested that patients with autosomal dominant inheritance of OAVS are more often bilaterally affected than patients with sporadic occurrence (Tasse et al., 2007 ; Vendramini-Pittoli and Kokitsu-Nakata., 2009), and rarely present extracranial abnormalities (Vendramini-Pittoli and Kokitsu-Nakata., 2009). The evidence, so far, does not suggest that the familial OAVS phenotype is different from the sporadic OAVS cases. Later, in 2011, Farra et al. identifyied a family where the ocula-auriculo- vertebral spectrum was characterized by microtia, mandibular hypoplasia, sacral vertebral defects, heart and brain, and with an apparent autosomal recessive inheritance (Farra et al., 2011) (Figure 2).

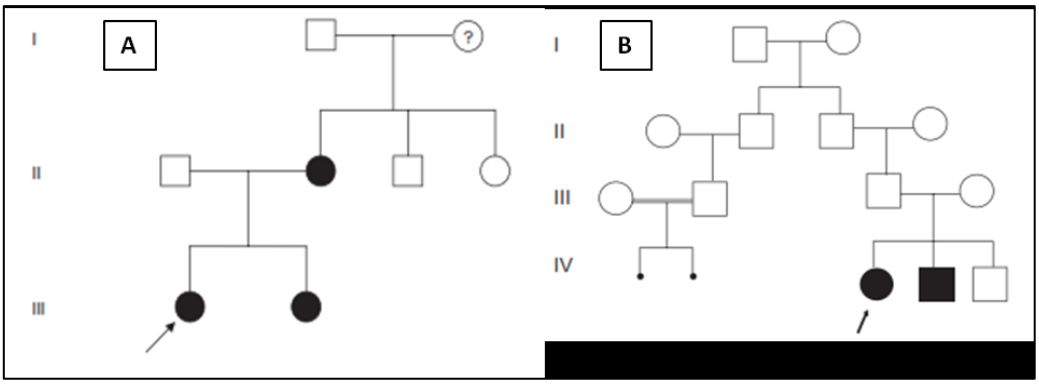


Figura 2. Examples of autosomal dominant and autosomal recessive families with OAVS reported in literature (Tasse et al., 2007 ; Farra et al., 2011).A) VI-1_Female patient with facial asymmetry, diaphragmatic hernia, encephalomeningocele, and preauricular skin tag. VI-2_Her sibling presented with facial asymmetry and abnormal ears. B) III-1_Female patient with hemifacial microsomia, microtia, preauricular tags. III-2_ Sister of III-1 presented with preauricular skin tags on the right side, mild asymmetrical mandibular hypoplasia. II-2 showed phenotypic expression of OAVS with scars of previously removed preauricular pits on both sides, right-sided hemifacial microsomia and a highly arched palate. I-2 The maternal grandmother showed that the position of her right ear and right palpebral fissure were higher than that on the left side and a mild unilateral lower jaw hypoplasia on the right side.

A number of chromosome abnormalities have been reported to be associated with OAVS including del(5p), monosomy (6q), del(8q), trisomy 18, ring chromosome 21, del(22q), dup(22q), trisomy 22 and 47,XXY. In addition, there are also reports of chromosomal mosaicism including trisomy 7(mosaic) and trisomy 9 (mosaic) which may account for localized features and low recurrence risk (Josifova et al., 2004). Among these, a terminal deletion of 5p

was reported in 8 unrelated patients, suggesting that it may harbor a gene or genes involved in OAVS (Ala-Mello et al., 2008 ; Josifova, et al., 2004 ; Tasse et al., 2007 ; Wang and Khan, 2010). Rooryck et al. performed high density oligonucleotide array-CGH on 86 OAVS patients and identified in 11 patients 12 novel genomic rearrangements (4 deletions and 8 duplications) ranging in size from 2.7 kb to 2.3 Mb, confirming genetic heterogeneity of OAVS (Rooryck et al., 2010). The best evidence for the possibility of a single gene having a major effect comes from familial cases, where the condition appears to segregate in a dominant manner (Kelberman, et al., 2001 ; Tasse, et al., 2007 ; Vendramini-Pittoli and Kokitsu-Nakata., 2009), with variable penetrance and phenotypic expression within and between families. A high lod score has been found for the 14q32 genomic region which harbors the Goosecoid gene (Gsc) (Kelberman et al., 2001). Recently, Huang et al., used bead cheep analysis on a three-generation family with autosomal dominant inheritance and reduced penetrance and found a significant load score on chromosome 15q26.2-q26.3(Huang et al.,2010). More recently, Ballesta-Martinez et al., described a family with clinical diagnosis of OAVS and autosomal dominant inheritance pattern in which they detected a 14q23.1 duplication of 1.34 Mb in size which segregates with the phenotype. This region contains *OTX2*, which is involved in the development of the forebrain, eyes, and ears, and appears to be a good candidate gene for OAVS (Ballesta-Martinez et al., 2013). Ou et al., had previously described a father and son with clinical features of OAVS and branchiootorenal (BOR) syndrome and the detection of an 11.79 Mb duplication of chromosome 14q22.3-q23.3 including *SIX1, SIX6*, or *OTX2* genes. He proposed that the increased dosage of genes included in the duplication might be responsible for the BOR and OAVS-like features in this family(Ou et al.,2008). This finding, together with previous findings supports the important role of 14q in OAVS phenotype and narrows down the candidate region to 14q23.1. Although a complex disorder, there is strong evidence to suggest a major genetic determinant in some cases of this condition. Anomalies in 22q have been frequently documented in patients with OAVS, particularly the 22qter deletion (Herman et al.,1988), 22q11.2 deletions (Xu et al., 2008 ; Digilio et al.,2009 ; Tan et al.,2011), the 22q11.1-q11.21 (Cat-eye) region(Quintero-Rivera and Martinez-Agosto., 2013 ; Torti et al.,2013), and a partial 22 trisomy (47,XX,+der(22)t(11;22)(q23;q11)), which duplicates the

22q11 region (De Ravel et al., 2001). Chromosomal mosaicism for trisomy 22(De Ravel et al., 2001) has also been described, making this region a good candidate for some cases of OAVS. More recently, array-CGH analysis identified 22q11 dosage anomalies in 10 out of 22 index cases screened hypothesizing that the 22q11 locus may harbour genes that are important in aspects of the regulation of craniofacial symmetry and 1st and 2nd branchial arch development (Beleza-Meireles et al., 2015). Interestingly, a central role of Crkl, a gene located in the 22q11 syndrome region, has been demonstrated in regulating signalling events in the developing of pharyngeal arches, with potential contribute to craniofacial dysmorphism. In fact, an altered retinoic acid and endothelin signaling has been evidenced in a *Crkl* mutant mouse. These two signalling pathways play an important role in the migrating and differentiation of neural crest cells in the branchial arches during embryogenesis (Miller et al., 2014).Among several published subtelomeric deletions in 20q13.33, a small heterozygous deletion including only *PCMTD2* and *MYT1* genes was described in a girl presenting with severe mental retardation and mild dysmorphic features (Kroepfl et al., 2008). More recently, in a study of 2016, Lopez et al., screened a cohort of 169 OAVS patient by whole exome sequencing (WES) identifying two mutation in the myelin transcription factor 1 (*MYT1*). A de novo nonsense mutation was found in a patient with facial asymmetry, preauricular and jugal tags, small and dysplastic ear and moderate conductive hearing loss with stenosis of the external auditory canal, epibulbar dermoid, and vertebral malformation. Furthemore a missense mutation was found in a patient and in his father both affected by OAVS. The proband presented a more severe phenotype with craniofacial anomalies with the presence of a sacrococcygeal dimple, pyloric stenosis and lumbar dysraphism without vertebral anomalies whereas his father show a mild phenotype characterized by a progressive bilateral sensorineural hearing loss diagnosed at the age of 25, a myopia diagnosed during adolescence and a thoracolumbar scoliosis with right convexity without vertebral malformation (Lopez et al., 2016). Functional studies by transient knockdown of myt1a, homologue of *MYT1* in zebrafish, led to specific craniofacial cartilage alterations. Treatment with all-trans retinoic acid (RA), a known teratogenic agent probably causing OAVS, led to an upregulation of cellular endogenous *MYT1* expression. Additionally, cellular wild-type *MYT1* overexpression

induced a downregulation of RA receptor β (RARB), whereas mutated *MYT1* did not (Figure 3).

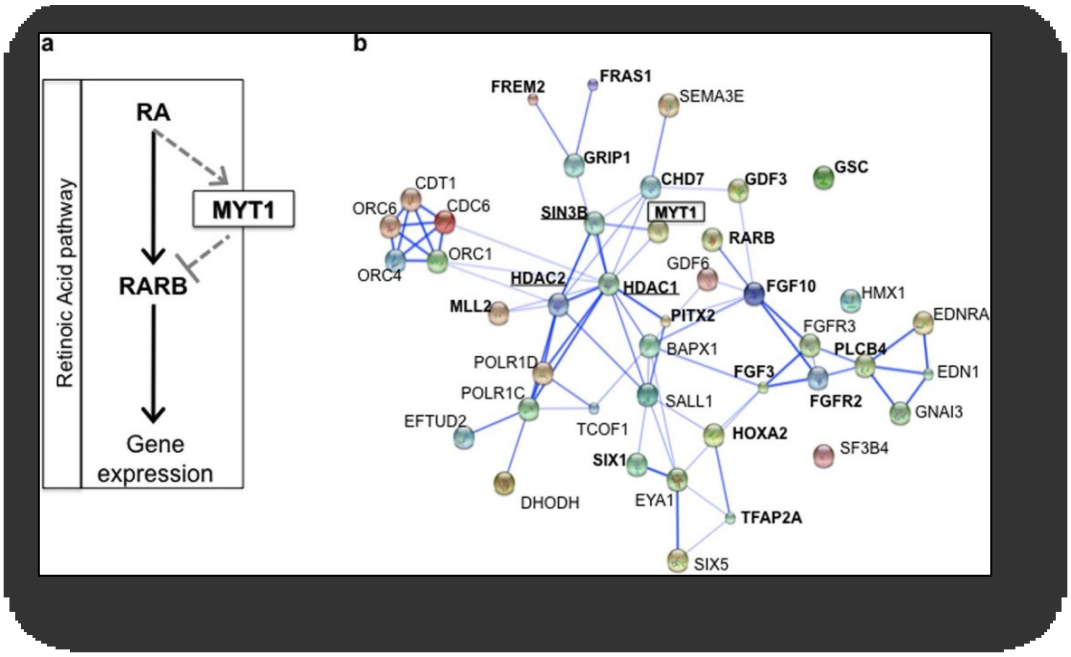


Figure 3 Myelin transcription factor 1 (*MYT1*) is involved in retinoic acid (RA) pathway and belongs to craniofacial disorders causative genes network. (A) Schematic model depicting *MYT1* action on RA pathway. Grey and dashed arrows link RA to *MYT1* overexpression and *MYT1* inhibition of RA receptor ß (RARB) expression as shown in the present study. (B) Network of protein-protein interactions is shown, produced by STRING software (http://www.string-db.org/), including *MYT1* (framed) and its closest partners *SIN3B, HDAC1* and *HDAC2* (underlined) along with craniofacial disorders causative proteins characterised in human and animal models. Thicker lines represent interactions of higher confidence. Proteins in bold have been reported in the literature as related to RA.

1.3a Murine models

Studies in mouse models, have also confirmed the involvement of genetic factors in the pathogenesis of OAVS.

Initially, in 1994, some transgenic mice caused by an insertional mutation on chromosome 10 produce a phenotype in hemizygous mice resembling HemiFacial Microsomia (HFM) in humans , including microtia and abnormal bite occlusion , secondary hypoplasia of the second and third branchial arch accompanied by a hemorrhagic event (Naora et al., 1994) (Figure 4).

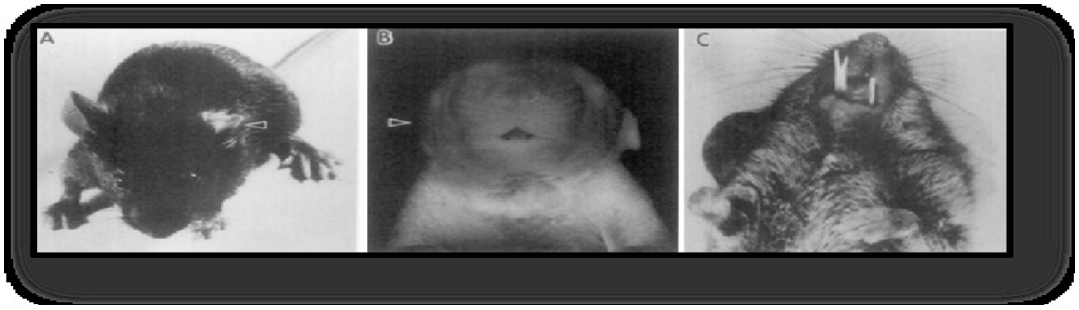


Figure 4. Phenotypes of transgenic line HFM. Microtia left (A), unilateral anotia dx (B) and abnormal dentition (C) (Naora et al 1994)

Meanwhile the mouse models with Otx2 +/- genotype showed a wide spectrum of craniofacial anomalies that included micrognathia, agnazia, anophthalmia and a narrowing of the head with lacking involvement of headset (Matsuo et al., 1995).

Additional evidence were later found by studies on mice with mutations in *ZIC3*, transcription factor involved in the lateralization during development. These mouse models developed multiple birth defects including: abnormalities of the skeleton/ribs, craniofacial abnormalities, left-right axis defects, congenital heart defects, alteration of the hyoid bone and laryngeal cartilage abnormalities (Zhu et al., 2007).

Some mouse models created by Minoux and colleagues in 2013, allowed to evaluate the expression of homeobox *Hoxa2* gene in mesenchymal cells derived from neural crest cells of the first branchial arch, where normally this gene is not expressed. As a result of this expression, mice showed specular earphones duplication; this observation linked the expression of *Hoxa2* with the development of the second branchial arch. Subsequently, the research group of Cox, in 2014, considering the results reached by the expression of ectopic *Hoxa2* in mice, noticed that there were both duplicated and specular auricular pinnae and both some small appendixes, just as also observed in patients with a OAVS phenotype, suggesting that the altered expression of the gene *Hoxa2* could be involved in these anomalies , focusing on some downstream genes such as *Hoxa2* as *Bmp4, Bmp5* and *Twsg1* (Minoux et al., 2013). None of these genes is specific molecular interpreter of the second branchial arch

development, rather they are necessary to the proliferation and differentiation of the auricular cartilage and other cell types. Consistently with what has been said previously, each of these genes when silenced, in mouse models, originates simple microtia phenotypes (Di Leone et al., 1998 ; Minoux et al., 2013 ; Petryk et al., 2004).

1.4 Branchial Arch Syndromes

OAVS involves primaryly the derivatives of the first and second pharyngeal arches, so it has been proposed that the aetiology and mechanisms of OAVS are related to the development of these structures. The first branchial arch is involved in the development of the face. During the 4th week to 8th week of gestation, the frontonasal prominence gives rise to the median facial structures.

The paired maxillary and mandibular prominence develop into the lateral facial structures. Small hillocks develop at the dorsal end of the first and second branchial arches from the 24th day of gestation. These hillocks gradually fuse to form the pinna of the external ear. The second branchial arch enlarges during the 5th week, forms the mandibular prominence and overgrows the 3rd and 4th arches. Both arches will develop into nerves, muscles, ligaments and skeletal structures, through a complex but poorly characterised signaling network (Szabo-Rogers et al., 2010 ; Chai and Maxson., 2006 ; Minoux and Rijli., 2010).

Disregulation of these signaling pathways triggered by genetic or environmental factors constitute a potential source of facial maldevelopment. The morphogenesis of the pharyngeal arch derivatives depends on continuous and reciprocal tissue–tissue interactions. One of the key features of craniofacial development is the formation of cranial neural crest cells, which migrate ventrolaterally as they populate the craniofacial regions. Disturbances in the specification, migration, proliferation, survival and ultimate fate determination of the cranial neural crest cells have been proposed as a possible mechanism for OAVS (Szabo-Rogers et al., 2010 ; Chai and Maxson., 2006 ; Minoux and Rijli., 2010). Manifestation and severity of the defect will thus depend on how the expression and activation of genes and proteins have been shifted during facial development.

1.4a Molecular mechanisms of patterning in the branchial arches

Appropriate epithelial-mesenchymal signaling is essential for proper development of the pharyngeal arches (Figure 5). Genetic analyses in mice provide evidence that numerous homeobox genes, including *Msx, Dlx*, goosecoid (*Gsc*), and *Prx*, and other transcription factors, such as *Hand2*, are expressed in pharyngeal arch mesenchyme and play essential roles in development of PA1 (Graham., 2003 ; Richman and Lee., 2003). Homeobox genes are a large group of genes that encode transcription factors responsible for regulating the expression of downstream target genes. The homeobox is a highly conserved 180 base pair sequence originally discovered in the homeotic selector genes of the fruitfly *Drosophila melanogaster*. Homeotic genes are a family of master regulatory homeobox genes, ultimately responsible for specifying segment identified along the anterior–posterior axis of the fly's developing. The vertebrate homologues are the Hox genes and these genes specify the vertebrate embryonic body axis during development (Akam.,1989). In addition, segment-specific combinatorial Hox gene expression in migrating neural crest cells is also responsible for generating diversity in the branchial arch system(Hunt and Krumlauf.,1991). However, Hox genes are not expressed in the first branchial arch and it has been suggested that this loss of Hox gene expression has been essential for the skeletal rearrangements to occur that are necessary for the development of a jaw (Cohn., 2002). Indeed, overexpression of Hox genes in the first branchial arch neural crest leads to a failure of differentiation into cartilage and bone (Couly et al., 1998 ; Grammatopoulos et al., 2000). If Hox genes are not directly responsible for patterning the first arch, then other mechanisms will exist to establish the positional fate of these ectomesenchymal cells.The ectoderm and endoderm that cover the first arch derivatives is characterised by distinct tempero-spatial regions of gene expression. Many of these genes encode secreted molecules and one candidate for playing an early role in patterning of the first branchial arch is the signalling peptide encoded by *Fgf-8*. Inactivation of mouse *Fgf8* specifically in PA1 epithelium revealed that Fgf8 promotes mesenchymal cell survival and induces a developmental program required for PA1 morphogenesis (Trumpp et al., 1999). It has been proposed that this specific expression of *Fgf-8* is involved in the early determination of polarity in the first branchial arch (Tucker et al., 1999). *Lhx-6* and *Lhx-7* are two LIM

homeobox domain genes that are characteristically restricted to the ectomesenchyme within the oral half of the first arch (Tucker et al., 1999 ; Grigoriou et al., 1998). However, this restricted expression domain is not an inherent property of these cells ; ectomesenchyme derived from the second branchial arch, which does not normally express either of these genes, can be induced to express both *Lhx-6* and *Lhx-7* when recombined with ectoderm from the oral surface of the first branchial arch (Tucker et al., 1999). *Fgf-8* is the most likely candidate as the endogenous inducer, its epithelial expression domain complements the ectomesenchymal domains of both genes and beads soaked in recombinant *Fgf-8* protein can induce both *Lhx-6* and *Lhx-7* expression in a concentration-dependent manner in isolated mandibular ectomesenchymal cultures(Tucker et al., 1999). *Goosecoid* (*Gsc*) is another homeobox-containing gene expressed within the ectomesenchyme of the first branchial arch, however, in contrast to *Lhx-6* and *Lhx-7*, *Gsc* expression is restricted to the aboral regions (Gaunt et al.,1993). Consistent with this restricted expression, mice with targeted mutations in *Gsc* have skeletal abnormalities of the mandible, including hypoplasia and malformations in Meckel's cartilage (Rivera-Perez et al., 1995 ; Yamada et al., 1995). Therefore, whilst *Fgf-8* is essential for normal patterning of the majority of the axis of the first arch, distally in the future incisor regions an alternative regulatory cascade seems to be important (Trumpp et al., 1999)

Distal-less genes incorporate a six-gene family of mammalian homeobox genes (*Dlx-1*, *-2*, *-3*, *-5*, *-6* and *-7*) that also exhibit highly nested domains of expression in the branchial arches during early development (Bulfone et al., 1993 ; Depew et al., 1999 ; Qiu et al., 1997). Within the mammalian genome, these genes are arranged in convergent pairs, with each pair having similar domains of expression (*Dlx-1/-2*; *Dlx-3/-7*; *Dlx-5/-6*) (Qiu et al., 1997). In particular, along the rostral-caudal axis of the branchial arches *Dlx-1* and -2 are expressed more-or-less continuously, whilst the expression domains of *Dlx-5/-6* and *Dlx-3/-7* are found to be progressively more restricted in a caudal direction (Depew et al., 1999 ; Qiu et al., 1997). The study of mice with targeted mutations in *Dlx* genes has suggested that a *Dlx* code of expression might be important in establishing inter-arch identity within the branchial region. Certainly, mice with loss of either *Dlx-1*, *Dlx-2* or *Dlx-1/-2* function exhibit progressively more severe anomalies in structures derived from the

more rostral regions of the branchial arches, in particular the maxillary process of the first branchial arch (Qiu et al., 1997 ; Qiu et al., 1995). Even though they are expressed in caudal structures, the loss of *Dlx-1/-2* does not seem to affect the patterning of these regions because of compensatory action by other *Dlx* genes, a finding confirmed by the presence of defects in regions of the mandibular arch of *Dlx-5*−/− mice (Depew et al., 1999 ; Qiu et al., 1997 ; Qiu et al., 1995 ; Acampora et al., 1999). Further, in mice lacking the function of both *Dlx-5* and *-6*, genes that are only expressed in more caudal regions of the branchial arches, a homeotic transformation is found to occur; these mice have a conversion of mandibular arch structures to maxillary (Depew et al., 2002). Thus, nested *Dlx* gene expression appears to play a fundamental role in establishing both the identity of different branchial arches and the identity of the maxillary and mandibular processes of the first branchial arch. Members of the Bmp family also have important roles in outgrowth of PA1 (Tucker et al.,1999). PA1 development appears very sensitive to the level of Bmp signaling during the initial outgrowth phase, and the level of Bmp signaling is tightly regulated by various factors (Massague and Chen., 2000).

The secreted protein Shh, a vertebrate ortholog of the Drosophila segment polarity gene, Hedgehog, is essential for normal development of many organs and is implicated as a cause of HPE (Holoprosencephaly). Shh is expressed in the pharyngeal arch epithelium and targeted disruption of Shh in mouse leads to near complete absence of craniofacial skeletal elements along with multiple organ defects (Chiang et al., 1996). Recent studies using chick and mouse embryos have suggested that Shh may play a role in NCC (neural crest cells) development and pharyngeal pouch patterning (Ahlgren and Bronner-Fraser., 1999 ; Jeong et al., 2004 ; Moore-Scott and Manley., 2005).

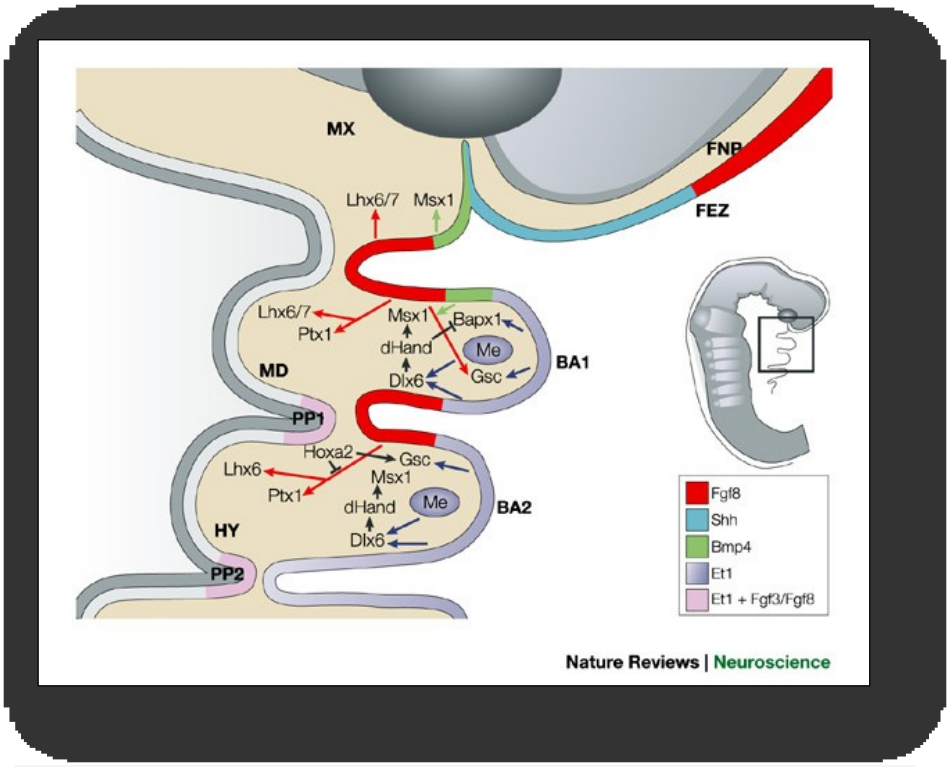


Figure 5.Molecular pathways involved in the specification of frontonasal, maxillary, mandibular and hyoid structures. A section through an imaginary plane is shown, which is mid-sagittal at the level of the frontonasal process (FNP) and parasagittal (slightly more lateral than the midline) at the level of the first and second pharyngeal arches (BA1 and BA2, respectively). The indicated genetic interactions are representative of data collected in mouse, chick or zebrafish. Some differences might exist in these pathways in different species. The ectodermal, endodermal and mesodermal localization of secreted signalling molecules is indicated, including bone morphogenetic protein 4 (Bmp4; green), sonic hedgehog (Shh; blue), fibroblast growth factor (Fgf8; red), endothelin-1 (Et1; violet), Fgf8/Fgf3 and Et1 (pink). Receptor molecules are expressed in the neural crest mesenchyme (yellow), though not exclusively, and they induce the activation and/or maintenance of the indicated transcription factors. In turn, the activity of transcription factors specifies the morphogenetic identity of structures both along the anteroposterior (inter-pharyngeal) and the dorsoventral (intra-pharyngeal) axes.

1.4b Differential diagnosis

The list of syndromes involving structures derived from the first and second pharyngeal arches is extensive (Gorlin., 2001) , but they are often associated with malformations derived from other embryological origins than these arches. First and second branchial arch syndromes manifest as combined tissue deficiencies and hypoplasias of the face, external ear, middle ear, the maxillary

and the mandibular arches and are the second most common craniofacial malformation after cleft lip and palate. Bilateral anomalies are present in 30% of these patients. Therefore, the presence of a familial history and a detailed clinical examination with biological evaluation may help to identify a known syndrome. Nowadays there is a tendency to group the clinical cases here described in a group of malformations called "LATERAL FACIAL MICROSOMIA" or "OTOMANDIBULAR DYSPLASIAS" (see Table 1).

The wide spectrum of otomandibular dysplasias makes them difficult to classify, but these deformities can be broadly considered as involving skeletal, auricular and soft tissue. Some patients with OAVS have clinical findings that overlap with other syndromes involving structures derived from the first and second pharyngeal arches. Usually, there is a tendency to consider as overlapping with OAVS fenothype, a lot of syndromes with known causative genes: Treacher Collins syndrome (Katsanis and Jabs., 2012) , Townes–Brocks syndrome (Kohlhase., 2007) , CHARGE syndrome (Lalani et al., 2006) , Branchio-oto-renal spectrum disorders (Smith., 2013) and the phenotypic spectrum associated with mutations in *EFTUD2* (Gordon et al., 2012 ; Voigt et al., 2013). The crux of the matter is that these conditions are distinctive and recognisable phenotypic entities. The presence of anal anomalies, for instance, points strongly towards Townes–Brock syndrome; the shape of the ear and semicircular canal abnormality in CHARGE syndrome are very characteristic, and individuals with *EFTUD2* mutations have microcephaly and may also have associated with esophageal atresia.

Treacher Collins syndrome

Treacher Collins syndrome (TCS, MIM 154500; TCS2, MIM 613717; TCS3, MIM 248390) is a rare congenital disorder of craniofacial development. TCS is characterized by hypoplasia of the facial bones, particularly the mandible and zygomatic complex, together with cleft palate, downward slanting of the palpebral fissures, and anomalies of the external and middle ear (Figure 6). Interestingly, there is a considerable degree of phenotypic variability in the severity and combination of these characteristic anomalies both between and within families (Dixon et al., 2004 ; Trainor et al., 2009). TCS occurs with an estimated incidence of 1:50000 live births and is primarily associated with autosomal dominant mutations in *TCOF1* (Wise et al., 1999). However, despite

extensive searches, the causative mutation in a subset of patients exhibiting classic features of TCS remained unidentified. The use of genome-wide copy number analysis in a child with TCS who was negative for a *TCOF1* mutation, led to the identification of a *de novo* 156-kb deletion within human chromosome 13q12.2 that resulted in deletion of the entire *POLR1D* gene (Dauwerse et al., 2011). *POLR1D* encodes a subunit of RNA polymerase I and III (Dauwerse et al., 2011). Subsequently, a further 242 individuals with classic features of TCS, but who were negative for *TCOF1* mutations, were sequenced, leading to the identification of additional *POLR1D* mutations (Dauwerse et al., 2011). In addition to 10 heterozygous nonsense mutations, seven heterozygous missense mutations located in exon 3 of *POLR1D* were discovered. Without exception, the missense mutations affected evolutionary-conserved amino acids in the RNA polymerase dimerization domain of *POLR1D*.Given the strong interaction between *POLR1D* (*RPAC2*) and *POLR1C* (*RPAC1*) in yeast (Yao et al., 1996) , *POLR1C*, which also encodes a subunit of RNA polymerase I and III,was sequenced leading to the identification of mutations in both *POLR1C* alleles in three affected individuals. In all cases, one mutant allele was inherited from each phenotypically unaffected parent, confirming autosomal recessive inheritance in a very small subset of TCS patients (OMIM248390) (Dauwerse et al., 2011 ; Vincent et al., 2016).

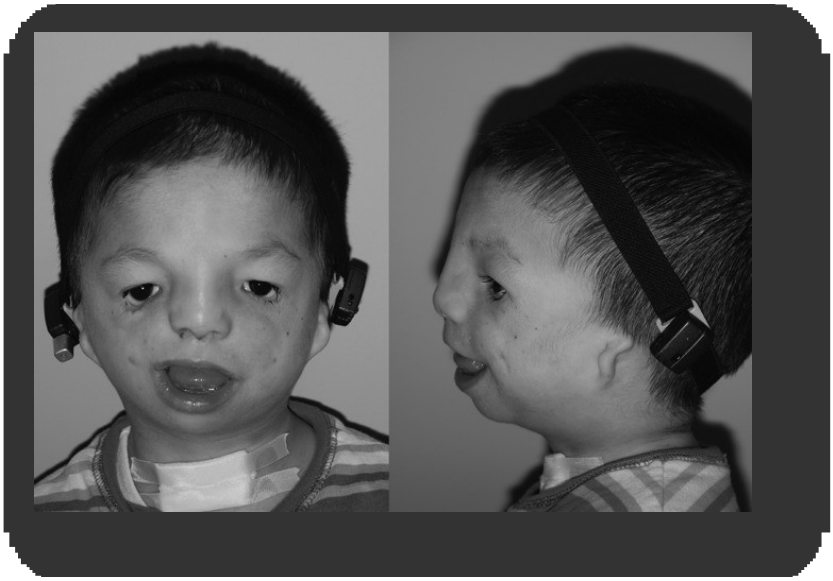


Figure 6.Characteristic findings of Treacher Collins syndrome include downward slanting palpebral fissures, lower eyelid colobomas, midface and zygomatic hypoplasia, microtia, and mandibular microretrognathia. Patients can be fitted with a removable bone-assisted hearing aid to help speech and language development prior to definitive implantation after ear reconstruction.

Townes–Brocks syndrome

Townes-Brocks syndrome (TBS; MIM 107480) is characterized by three main clinical signs: imperforate anus in '82% of patients, in 88% of patients were observed ear abnormalities (dysplasia of the upper helix and preauricular pits) associated in 65% of cases with sensorineural or conductive hearing loss, and malformations of the thumb.

Minor clinical signs showed renal abnormalities occurred in 27% of cases, cardiac abnormalities in 25% of patients and in 56% of cases, deformities of the feet (flat feet or overlapping fingers). Finally also genitourinary malformations (36% of cases have been reported) and intellectual disability in about 10% of cases. The gene responsible for the disease is *SALL1* (MIM 602218), located in the chromosomal region 16q12.1. Point mutations or deletions / duplications of the gene are responsible for 70% of cases of Townes-Brocks. *SALL1* encodes a transcription factor, which performs its function as a repressor during the early stages of embryogenesis. Functional studies have also shown that the promoter region binds directly to *SIX1* gene promoting its expression through a dose-

dependent effect and making this also a candidate gene to be tested in patients with an atypical clinical phenotype of BOR syndrome (Chai et al., 2006).

CHARGE syndrome

CHARGE syndrome is a pleiotropic disorder whose name is derived from the acronym summarizing its six clinical principal features: ocular coloboma, heart defects, choanal atresia, retardation of growth/development, genital anomalies, and ear anomalies/deafness (Figure 7). *CHD7* is the only gene mutation of which is known to cause CHARGE syndrome, and mutations are identified in approximately two-thirds of patients with a clinical diagnosis of CHARGE syndrome (Yasuda et al., 2016)

Various chromosomal rearrangements have been reported in rare patients with a CHARGE-like phenotype. These include balanced translocation between chromosomes 6 and 8(Hurst et al., 1991), unbalanced translocations involving chromosomes 2 and 18, 3 and 22(Clementi et al., 1991), partial trisomy of 19q with partial monosomy 21q (De Krijger et al., 1991), inverted duplication of chromosome 14 (14q22R q24.3) (North et al., 1995) and partial trisomy of 2q (Lev et al., 2000). Based in part on the inconsistent chromosomal aberrations in rare patients with CHARGE association, it is most likely that this condition is genetically heterogeneous. Within the group of children with CHARGE association, there is clearly a subgroup with distinctive clinical characteristics that appears to have a recognisable syndrome (Graham., 2001) Previously, a systematic scan for loss of heterozygosity using microsatellite markers in 10 such patients failed to identify a discernible submicroscopic deletion (Lalani et al., 2003). Although several candidate genes such as *PITX28* and *PAX29* have been investigated, no mutations have been identified in patients with CHARGE syndrome. Recent evidences demonstrate a *de novo* mutation in *SEMA3E* in an affected patient, identified upon mapping the translocation breakpoints in an unrelated individual with a *de novo* balanced translocation involving chromosomes 2 and 7 (Lalani et al.,2004) .

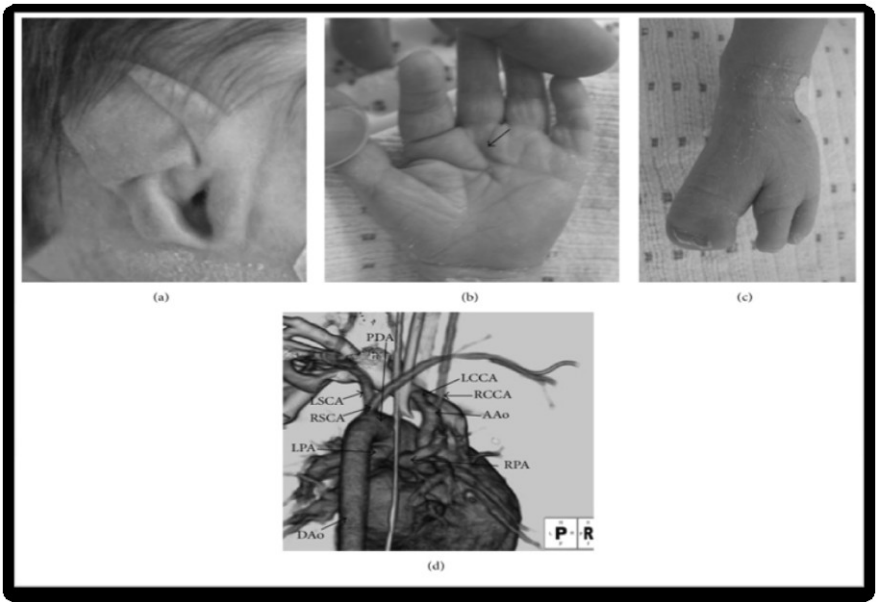


Figure 7:Dysmorphic features and cardiovascular malformations in Case 1. (a) "CHARGE ear." (b) Hockey-stick palmar crease (arrow). (c) Cleft foot. (d) 3D-CT angiography showed truncus arteriosus type A4, interrupted aortic arch type B, and aberrant origin of the right subclavian artery. AAo, ascending aorta; DAo, descending aorta; LCCA, left common carotid artery; LPA, left pulmonary artery; LSCA, left subclavian artery; PDA, patent ductus arteriosus; RCCA, right common carotid artery; RPA, right pulmonary artery; RSCA, right subclavian artery.

Branchio-oto-renal spectrum disorders

Branchiootorenal spectrum disorders comprise branchiootorenal (BOR) syndrome and branchiootic syndrome (BOS).

- BOR is characterized by malformations of the outer, middle, and inner ear associated with conductive, sensorineural, or mixed hearing impairment, branchial fistulae and cysts, and renal malformations ranging from mild renal hypoplasia to bilateral renal agenesis. Some individuals progress to end-stage renal disease (ESRD) later in life.

- BOS has the same features as BOR syndrome but without renal involvement.

Extreme variability can be observed in the presence, severity, and type of branchial arch, otologic, audiologic, and renal abnormality from right side to left side in an affected individual and also among individuals in the same family. BOR syndrome and BOS can be seen in the same family.The diagnosis of branchiootorenal spectrum disorders is based on clinical criteria. Molecular genetic testing of *EYA1* (BOR1, BOS1) detects pathogenic variants in approximately 40% of individuals with the clinical diagnosis of BOR/BOS.

Pathogenic variants can be detected in an additional 5% and 4% of individuals with the clinical diagnosis of BOR/BOS by molecular genetic testing of *SIX5* (BOR2) and *SIX1* (BOR3, BOS3), respectively. BOR syndrome and BOS are inherited in an autosomal dominant manner. The offspring of an affected individual are at a 50% risk of inheriting the pathogenic variant.

Prenatal testing for pregnancies at risk is possible if the pathogenic variant has been identified in a family member (Smith RJH et al,.1999 updated 2015 Oct 22).

Mandibulofacial dysostosis, Guion–Almeida

Mandibulofacial Dysostosis, Guion-Almeida Type (MFDGA, OMIM#610536, also called mandibulofacial dysostosis with microcephaly) is a multiple malformation syndrome characterized by progressive microcephaly, choanal atresia, cleft palate, mandibular hypoplasia, microtia, preauricular tags, conductive deafness, congenital heart and/ or thumb anomalies and developmental delay (Wieczorek et al., 2009 ; Lines et., 2012). Phenotypes of mandibulofacial dysostosis also overlap with other syndromes such as Pierre-Robin, Miller, Treacher Collins, and Nager syndromes, as well as oculoauriculovertebral (OAVS) spectrum disorders such as VATER association, CHARGE, Goldenhar and Feingold syndromes (Lines et al., 2012 ; Gordon et al., 2012 ; Need et al., 2012). MFDGA is inherited in an autosomal dominant manner and is caused by mutations in the *EFTUD2* gene. *EFTUD2* (OMIM#603892, elongation factor Tu GTP binding domain containing 2, also called U5 snRNP-specific protein, 116-KD) coded by *EFTUD2*, is a small nuclear ribo- nucleoprotein and is a component of the spliceosome complex which plays an important role in pre-mRNA splicing process (Fabrizio et al., 1997; Lines et al., 2012). Actually, 29 causative mutations have been identified in patients with MFDGA. These mutations are: missense (7/29), nonsense

(6/29), splicing (4/29), small deletion/insertions (8/29), and large deletion (4/29). The majority of the reported cases have a *de novo* heterozygous loss of function mutation (Lines et al., 2012 ; Gordon et al., 2012 ; Need et al., 2012).

Table 1 Differential diagnoses of OAVS

Diagnosis	Main clinical features	Gene	Inheritance
Treacher Collins syndrome OMIM 154500, 613717, 248390	Hypoplasia of the zygomatic bones and mandible External ear abnormalities frequently associated with hearing impairment Coloboma of the lower eyelid Absence of the lower eyelashes Preauricular hair displacement onto the cheeks Craniofacial involvement is generally symmetrical	*TCOF1* *POLR1D* *POLR1C*	Sporadic (60%) Autosomal Dominant(40%)
Townes–Brocks syndrome OMIM 107480	Imperforate anus Dysplastic ears (overfolded superior helices and preauricular tags) frequently associated with sensorineural and/or conductive hearing impairment Thumb malformations (triphalangeal thumbs, duplication of the thumb, preaxial polydactyly or hypoplasia of the thumbs) Renal impairment with or without structural abnormalities	*SALL1*	Autosomal Dominant
CHARGE syndrome	Coloboma of the iris, retina-choroid, and/or disc	*CHD7*	Autosomal Dominant

OMIM 214800	Unilateral or bilateral choanal atresia or stenosis Ear abnormalities (external ear malformation, ossicular malformations, Mondini defect of the cochlea and/or absent/hypoplastic semicircular canals) Cryptorchidism in males and hypogonadotrophic hypogonadism in both males and females Cardiovascular malformations Orofacial clefts Tracheoesophageal fistula Cranial nerve dysfunction		
Branchio-oto-renal spectrum disorders (branchio-oto-renal and branchio-otic syndromes) OMIM 113650, 610896, 602588	Malformations of the outer, middle and inner ear Conductive, sensorineural, or mixed hearing impairment Branchial fistulae and cysts, Renal malformations ranging from mild renal hypoplasia to bilateral renal agenesis. Branchio-otic syndrome has the same features as branchio-oto-renal syndrome but without renal involvement.	*EYA1, SIX5* and *SIX1*	Autosomal Dominant
Mandibulofacial dysostosis, Guion–Almeida—type OMIM 610536	Oto-facial abnormalities (acrofacial dysostosis) Oesophageal atresia	*EFTUD2*	Autosomal Dominant

	Thumb anomalies Intellectual disability Zygomatic anomalies Microcephaly		

1.5 Treatment considerations

The treatment strategy for patients should prioritize procedures that result in functional improvement while delaying those with cosmetic goals until growth maturity. The following functional categories may necessitate early intervention: airway, feeding, hearing, speech, vision, and socialization.

a) Airway

Maxillary and mandibular hypoplasia can contribute to a compromised airway. Choanal atresia or stenosis can compound this prob- lem,which may require immediate intervention ranging from observation with pulse oximetry and positioning to mandibular advancement with distraction osteogenesis, or tracheotomy

b) Feeding

An incompetent lip seal or cleft lip and or palate may compromise adequate nutrition. Considerations to cleft treatment algorithms or G-tube placement may be necessary.

c) Hearing

Pediatric otolaryngology consultation and formal audiology testing is required early in order to establish successful hearing.

d) Vision

Pediatric ophthalmologist should be consulted to evaluate for any extra ocular muscle (EOM) dysfunction or deficits in visual acuity. When the lateral and inferior orbital supporting structures are hypoplastic or missing the corneas are unprotected,which may necessitate earlier reconstruction of the orbital and zygomatic structures.

e) Speech

Correction of a cleft palate should follow the standard cleft treatment recommendations.

Secondary treatment strategy should take place at the end of skeletal maturity and include orthognathic surgery and zygomatic-orbital reconstruction. Malar and orbital reconstruction uses a full-thickness calvarium bone graft through a coronal incision and exposure. It is recommended that this procedure be performed after the age of 6 years, for skeletal maturity of the midface, as well as the ability to reconstruct the donor calvarium site with a local split-thickness calvarium graft. Maxillomandibular reconstruction is best approached with traditional orthognathic surgery at 13 to 15 years of age, at the time of early skeletal maturity(Figure 8). Le Fort I and rami osteotomies with sliding genioplasty are usually indicated. When the deformity results in absent ramus, condyle, or glenoid fossa, then reconstruction may be performed at the time of malar and orbital reconstruction. The use of costochondral bone graft to reconstruct the ramus-condyle is advocated. A second reconstruction with conventional osteotomies is almost certain to be warranted after skeletal maturity. As with conventional orthognathic surgery, nasal reconstruction should be performed as the final reconstructive procedure when indicated (Alfi et al., 2014).

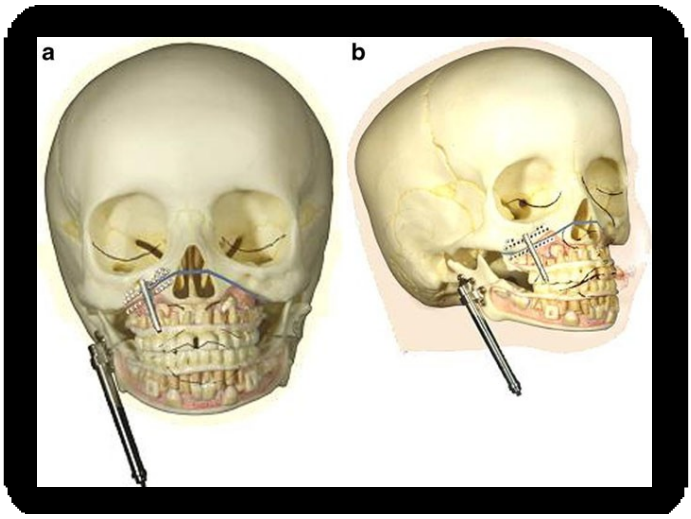


Figure 8. Schematic drawings of the application of two distracters on the maxilla and mandible in HFM show application of the maxillary and mandibular distractors after performing the Le Fort I osteotomy and the horizontal osteotomy of the mandibular ramus.

2. Aim of the project

OAVS is a complex and heterogeneous disorder that involves first and second branchial arch derivatives including maxillo-facial complex, kidneys, skeletal system and heart. It is a rare disease with a prevalence of about 0.3% in the general European population. Although the etiology of this disease is still unknown, a connection between OAVS and maternal diabetes or teratogens was mentioned. Embryonic hematoma formation or ectodermal non-disjunction early in development, was also suggested to be causative for OAVS. However, the presence of familial cases following autosomal dominant or autosomal recessive inheritance, as well as detection of several chromosomal aberrations, and the hemifacial microsomia mouse model, indicate that a significant number of OAVS cases may have a genetic basis. Genetic heterogeneity has been hypothesized and the identification in OAVS patients of many distinct microdeletions and microduplications throughout cytogenetic and array-based studies, confirm this hypothesis (Rooryck et al., 2010 ; Beleza-Meireles et al., 2015). With the advent of next generation sequencing (NGS) technologies, allowing investigation of multiple genes through one single reaction, the genetic basis of several Mendelian diseases characterized by genetic heterogeneity have been solved. Recently, NGS has been applied to the analysis of the whole exome of five OAVS patients. The authors identified two mutations in *MYT1*, a candidate gene involved in the retinoic acid pathway (Lopez et al., 2016). In our laboratory we have collected a large OAVS cohort.

This clinically-well characterized cohort has been already studied for the presence of microdeletion and microduplication by SNP array, showing that part of the patients carry de novo microrearrangements. Starting from this data, my research project is focused on the use of high-throughput targeted and exome sequencing approaches to search for novel gene(s) causing OAVS. Moreover, this kind of approach allows further insight into the pathways underlying craniofacial development with implications for other similar disorders as isolated microtia or syndromes of the first and second pharyngeal arches. For this purpose I have: 1) used target sequencing [Truseq Custom Amplicon (TSCA)] technology to analyze a panel of candidate genes in a group of selected OAVS individuals (n=65); 2) used Nextera XT technology in order to study the contribute of *MYT1* gene's mutations in our cohort of OAVS

patients (n=73); 3) scanned for mutations *HOXA2* gene in a family with isolated microtia; 4) analyzed the entire exome of a family with four affected individuals, where OAVS segregated as Mendelian autosomal dominant trait in two different branches[Ion Proton PCR based enrichment kit (Life Technologies)]. Identification of the underlying causes of OAVS will provide the basis for appropriate genetic testing, clinical management, genetic counseling, with improvements in risk assessment, prognosis and prevention, and for the development of new therapeutic approaches.

3. Materials and Methods

- Patients

Over these three-year's project, I have collected peripheral blood's samples of 55 individuals (23 patients and 22 non affected family members) expanding the cohort of previously available samples up to 232 individuals including 118 patients (51 females and 67 males) with oculo-auriculo-vertebral spectrum (OAVS) and 114 non affected family members for a total of 113 indipendent family. Among the 118 patients with OAVS, 105 were sporadic cases and 13 were familiar forms belonging to eight independent families, including a family with a pair of monozygotic twins with discordant phenotype. All participants in this study were selected based on the minimum diagnostic criteria described by Tasse et al., 2005, which include the presence of facial asymmetry associated with ear malformations and / or preauricular pits or fistulas. The OAVS cohort include a total of 20 patients with Goldenhar syndrome, the most severe form of OAVS, where in addition to craniofacial abnormalities might be present ocular abnormalities (such as coloboma or epibulbar dermoid), vertebral anomalies, cardiac and genitourinary malformations.

- DNA extraction

Genomic DNA was extracted from peripheral blood using manual kit (Macherey-Nagel) according to the manufacturers instructions. DNA concentration was assessed throughout Qubit™ fluorometer(Invitrogen) and purity parameters (260/280=1.8/2 ; 230/280=1.8/2) evaluated using the NanoDrop1000 Spectrophotometer (Thermo Scientific, Waltham, USA).

- SNP-array approach

In the first place, given a lot of evidences of genomic rearrangements identified in OAVS patients, samples have been evaluated, using SNP-array approach in order to determine the prevalence and the types of genomic micro-rearrangements and check for recurrent abnormalities associated with specific phenotypes. 64 OAVS samples previously collected, were already analyzed

with SNP Chip 6.0 (Affymetrix). Subsequently, I have collected other 23 OAVS samples enlarging the cohort of patients analyzing them with Cytoscan HD chip (Affymetrix).

Data analysis was conducted considering all the duplications and deletions (CNV, copy-number-variants) with a size greater than 75 Kb, and including at least 25 probes. The identification of CNVs, candidate genes and genomic regions associated with OAVS was conducted using different databases of genomic variants including "Genomic Variants" (http://projects.tcag.ca/variation/), "UCSC Genome browser "(http://www.genome.ucsc.edu/), and" DECIPHER " (http://www.sanger.ac.uk/PostGenomics/decipher). All CNVs containing genes coding for proteins and/or microRNA and all CNVs lacking of genes, but flanking genes (far no more than 1Mb from the rearrangement) as well as CNVs that were not present in our laboratory's database of internal genomic variants and/or absent in control populations listed in the previous reported database of genomic variants, were validated by quantitative real-time PCR assays, using a protocol based on detection with SYBR-Green molecule (Applied Biosystems) using real-time PCR ABI 7900 (Applied Biosystems).

3.1 NGS approach

a) Targeted sequencing

Starting from the genes involved in the rearrangements identified by SNP array analysis, genes belonging to protein functional networks involved in craniofacial development, genes related to disorders with clinical features overlapping with OAVS, I have used bioinformatics prediction software (BIOGRID, GeneMania, String, IPA) to built a network of 78 genes used in order to draw a targeted panels based on an amplicons' approach (TruSeq Custom Amplicon (TSCA), Illumina San Diego CA) (Figure 9).

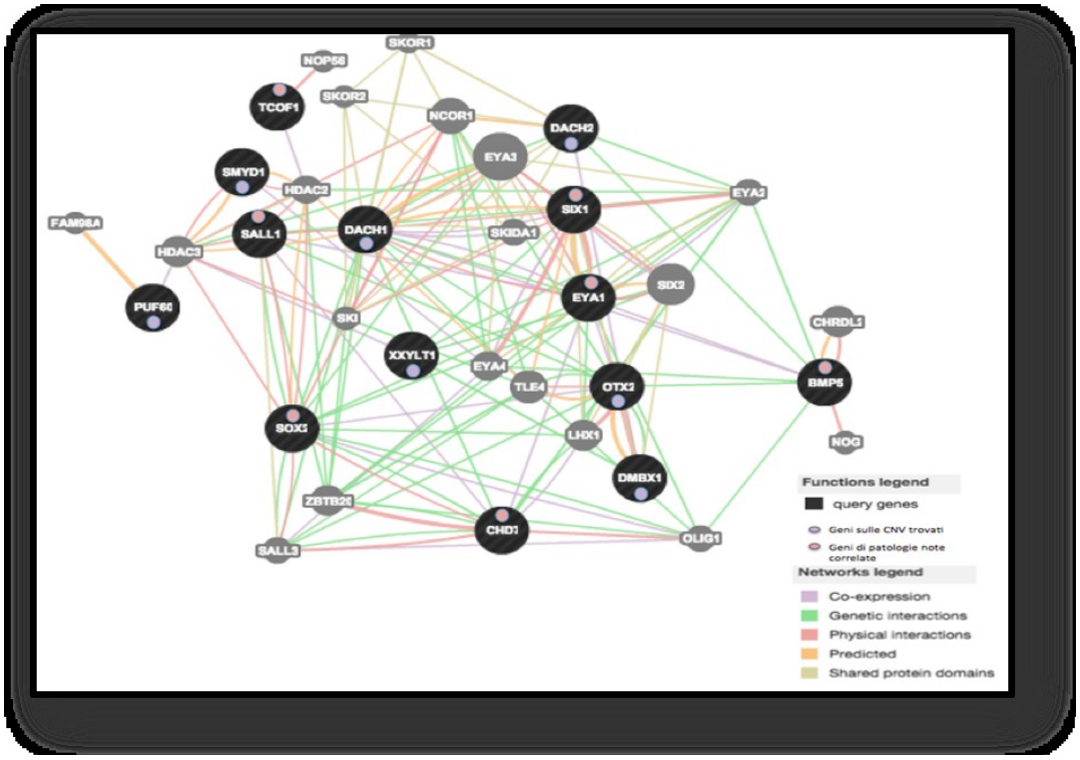


Figure 9. Genes network built considering those genes involved in the genomic rearrangements detected troughout SNPs array approach, genes belonging to protein functional networks involved in craniofacial development; genes related to disorders with clinical features overlapping

The design of the probes was performed using the "Design Studio software" (Illumina), available online. The probes were designed in the exonic regions and in the UTR regions of selected genes considering several parameters including the GC content, the specificity for a particular gene, the interaction between the probes and the "coverage". The first panel had a target region of 242.944 kbp, divided in 1003 amplicons and included 98% of the target sequence. The second panel covering a target region of 206.055 kbp, was divided into 889 amplicons and included 97% of the target sequences (Figure 10).

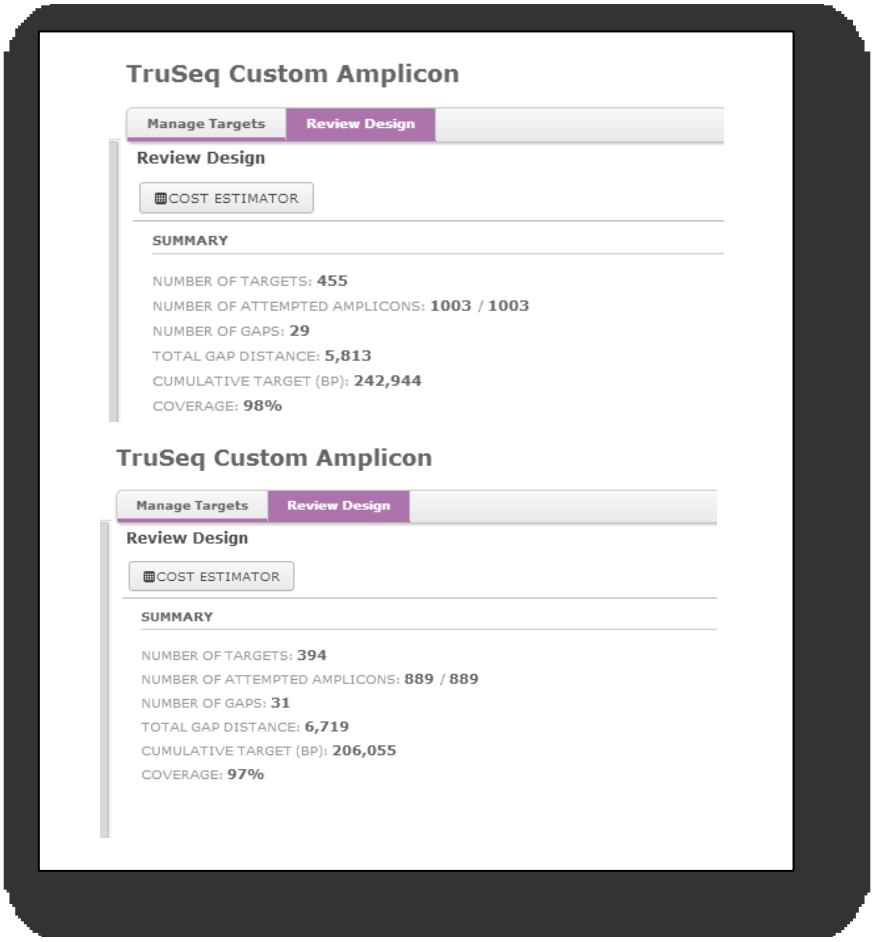


Figure10. Two targeted genes panels designed troughout "Design Studio software" (Illumina), available online. The first panel had a target region of 242.944 kbp, divided in 1003 amplicons and included 98% of the target sequence. The second panel covering a target region of 206.055 kbp, was divided into 889 amplicons and included 97% of the target sequences.

65 OAVS samples have been analyzed using 250 ng of DNA as input and the enrichment kit "TruSeq Custom Amplicon v1.5" (Illumina, San Diego, California), following the Illumina's user guide (Figure 11).The produced libraries were sequenced using the "MiSeq Reagent Kit-600 v3", with a" theoretical throughput " of about 15 Gb for run. The sequencing analysis was carried out paired-end throughout a second generation sequencer "MiSeq Desktop Sequencer" (Illumina). Paired-end sequencing allows users to sequence both ends of a fragment and generate high-quality, alignable sequence data. Paired-end DNA sequencing also detects rearrangements such as small insertions and deletions.

The data obtained were analyzed with the software "MiSeq Reporter". The "reads" are aligned considering the reference sequence Homo Sapiens GRCh37/hg19 using two software (BWA and BOWTIE2). SNPs, insertions/deletions and annotations were identified using the Genome Analysis Toolkit software (GATK3.6). Bioinformatic analysis identified a total of about 400 variants for each analyzed sample. Prioritization was performed by selecting the exonic and splicing variants, excluding the synonymous ones. All the variants that had a frequency greater than 1% in certain population's database available online such as dbSNP143 (https://www.ncbi.nlm.nih.gov/projects/SNP/) Go-Exp (http://evs.gs.washington.edu/EVS/) and Exac (http://exac.broadinstitute.org/) were eliminated. Troughout this filtering criteria, it has been selected an average of four variants for each sample whose pathogenicity was assessed using 13 pathogenicity predictors including SIFT, Polyphen, Mutation Assessor and CADD. All variants identified, have been confirmed subsequently by Sanger sequencing. Where parents of proband were available, it has been studied the segregation of variants identified also in unaffected members.

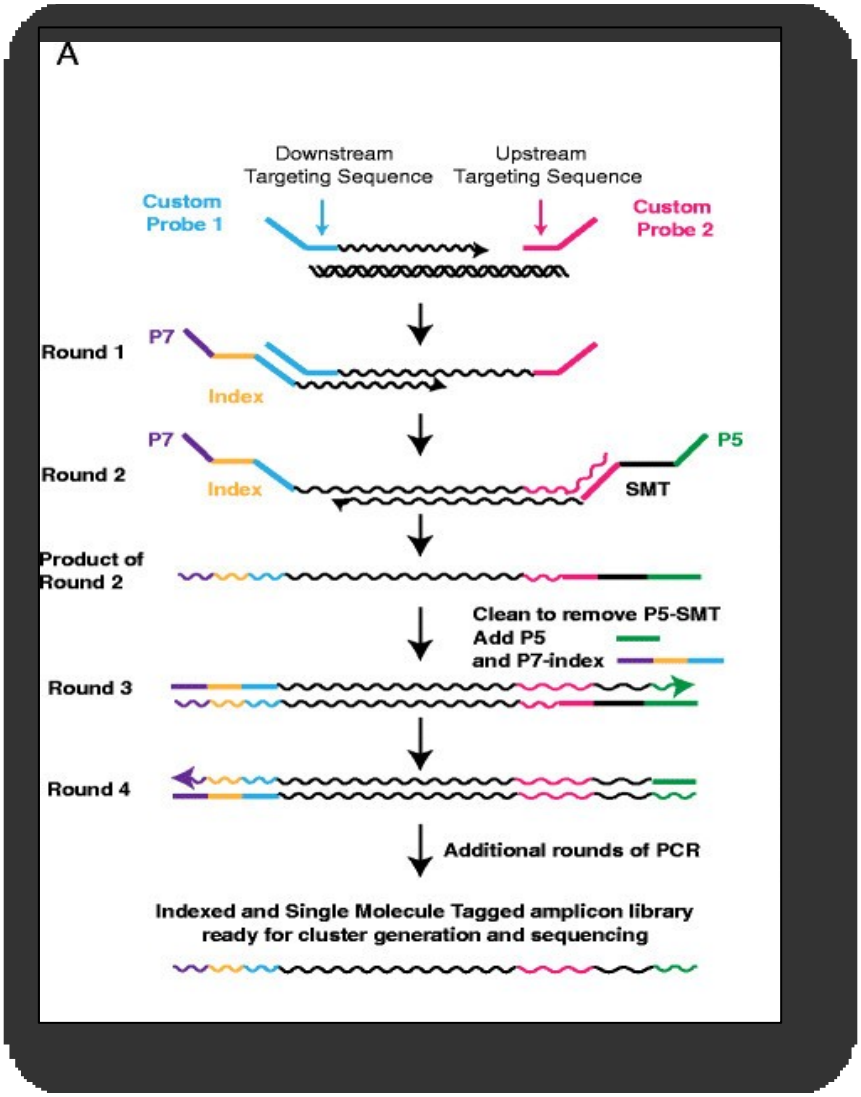


Figure 11: Adaptation of Illumina TruSeq Custom Amplicon Kit to allow for single molecule tagging. (A) Schematic of method showing amplification of target DNA using custom probes and flanking primers. The P5-SMT primer is the same as the standard P5 index primer, but contains a degenerate 12 N-mer sequence in place of the index. The incorporation of an Ampure Bead size selection step after two rounds of PCR removes unused P5-SMT, and the P5 primer is added to facilitate downstream amplification.

b) Nextera XT sequencing

Following the identification of two OAVS patients with point mutations in the *MYT1* gene involved in the retionoic acid pathway (Lopez et al., 2016), I have screened 73 OAVS samples selected from our cohort by Nextera XT DNA

Library Preparation protocol (Illumina). All samples have been included only if they showed the minimum diagnostic criteria described by Tasse et al., 2005 and an high quality of DNA. The Nextera XT sequencing protocol enable to sequence a wide range of input samples, including PCR amplicons greater than 300 bp. All the 21 coding exons of *MYT1* gene (NM_004535) including exon-intron boundaries and the 5' and 3'UTR regions were amplified to obtain amplicons greater than 300 bp (see Table 2). Genomic DNA was amplified by PCR under the following conditions: an initial denaturation of 5 min at 95°C, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing depending on amplified exon for 30 sec ,extension at 72°C for 30 sec and a final extension at 72° for 7 min. PCR was performed using GoTaq® DNA Polymerase protocol. Subsequently all the amplicons were multiplexed obtaining a starting PCR 96 well plate with 73 wells where each well contained one single DNA sample amplifyied for all 21 Myt1's exons with in addition UTRs regions.

Table 2 Primers and Conditions for Genomic Amplification of *MYT1*

MYT1	SIZE (pb)	FORWARD	REVERSE	T°
EX1 5' UTR	490 pb	CAGCAGATCCATCTTTCT GATGCT	CCTTCATCTTAGAAATTA ACTCT	58
EX2 5' UTR	476 pb	GTTGTCAGTGGGAACCA ACA	AAAGTCGCCTCCATGCTT CA	60
EX3	553 pb	TGCTGATGGGACACTTC CAAG	TGAGCCTTGCTACTCCCA CT	62
EX4	709 pb	TGTCTGAGGTGGAGCAT CTGTC	TTCTCTCATCTCATCCCC GAAC	66
EX5	793 pb	TTCTGTGCTTTGGACTTG GGAC	TCCCCACAGACTCATCCA GT	64
EX6	488 pb	ACTGGATGAGTCTGTGG GGA	ATGCCTTGTTCTCAGGAA GG	60
EX7a	436 pb	TGGTGGGTGTGTTGGGG ACT	CTGCTTGCTGGACTCCTC ACT	64
EX7bc	837 pb	TGGTGGAAGAGGACTTG GGC	AGGGCTCTCAGCCTGCAT CTG	62
EX8	572 pb	CTGGTTTCTTCAGGGTCT CCT	TCTGTTACTGTGGGCTCT GAC	62
EX9	799 pb	GGTCAGAGCCCACAGTA ACAGA	AGAAGGGTAGCACACAG GGAG	64+d mso
EX10	548 pb	GACTGGGCAGCTTGTAC TGTG	GAGACTCTGTCCTGTGTG GCT	66
EX11	493 pb	CCCAGCTTTAGTGGTGG AATA	AGAGTTTCCCTCCCTAGT AGC	62

EX12	530 pb	CCCCACTGACTTGTCTGCAT	CCTAACACGTGGGAACTTGTGT	64
EX13	614 pb	ATGGGAAATCAGGAAATGGCAG	AGCTGCTCAGAAGATGAAGGA	62
EX14	497 pb	CTGAAGCAGGGGAGGGATC	TCGAGGTGGTCAGGACAGGT	64
EX15-16	573 pb	AAGGACTGAGTGGAGGAGGG	AGAGCAGCCCCAGCCAAGCA	64
EX17	620 pb	CAAAGCACTGAACCCCTGAGAT	TGGGAATGGACTCTAGCTGTC	62
EX18	490 pb	TCACAGAGCTTGAGGGGAGAG	GGGCTCCTATCTTCACAGGT	62
EX19	424 pb	TGTGTCTGGCTTGAGAGAGT	CCTTGAGCAACTCTGGTCAC	60
EX20-21	987 pb	GACCCTGGGCTGGTGAATG	TCAGCCCTGACAGCAACCTT	62
EX22	405 pb	AGAGGGTTGTGAGAGGCAG	AACCCACCACAGCCAACAG	60
EX23	500 pb	GGATGGCTGCAGAGAGGAACG	GCCCCTATGCACACAGATAGG	66
3'UTRa	567 pb	ACTCACAGTGACTTCCCGTTTG	AGACATTAGGCTGATTTTCAGGAG	66
3'UTRb	666 pb	CAGTTTTGTGTTAAGGGTGGA	CGTTAACATAAGAGGCTGTGA	60
3'UTRc	446 pb	GTGACTGCAATTCCAAGTTAGTA	TCTTTCTCTCCATTAGACCAAG	62
3'UTRd	534 pb	CTTGGTCTAATGGAGAGAAAGA	CAAAAGGAGATACTGGCTAAGC	60

Starting from 0,3ng of PCR products, this protocol comprises a first step where Nextera transposome is used in order to tagment gDNA, which is a process that fragments and then tags the DNA with adapter sequences in a single step. This step amplifies the tagmented DNA using a limited-cycle PCR program. The PCR step adds Index 1 (i7) adapters and Index 2 (i5) adapters and sequences required for cluster formation.The second step is a CleanUp step using AMPure XP beads to purify the library DNA and provides a size selection step that removes short library fragments.The third step consists of checking libraries, by running 1 µl of undiluted library on an Agilent Technology 2100 Bioanalyzer using a High Sensitivity DNA chip. Finally, after libraries normalization to ensure more equal library representation in the pooled sample, they were pooled combining equal volumes of normalized libraries in a single tube (Figure12). After pooling, it is necessary dilute and heat-denature the library pool before loading libraries for the sequencing run

using the "MiSeq Reagent Kit-300 v2", with a" theoretical throughput " of about 8 Gb for run on Mi-Seq sequencer. The sequencing analysis was carried out paired-end and 151 cycles. The data obtained were analyzed with Illumina Variant Studio Data Analysis Software. Prioritization was performed as described below.

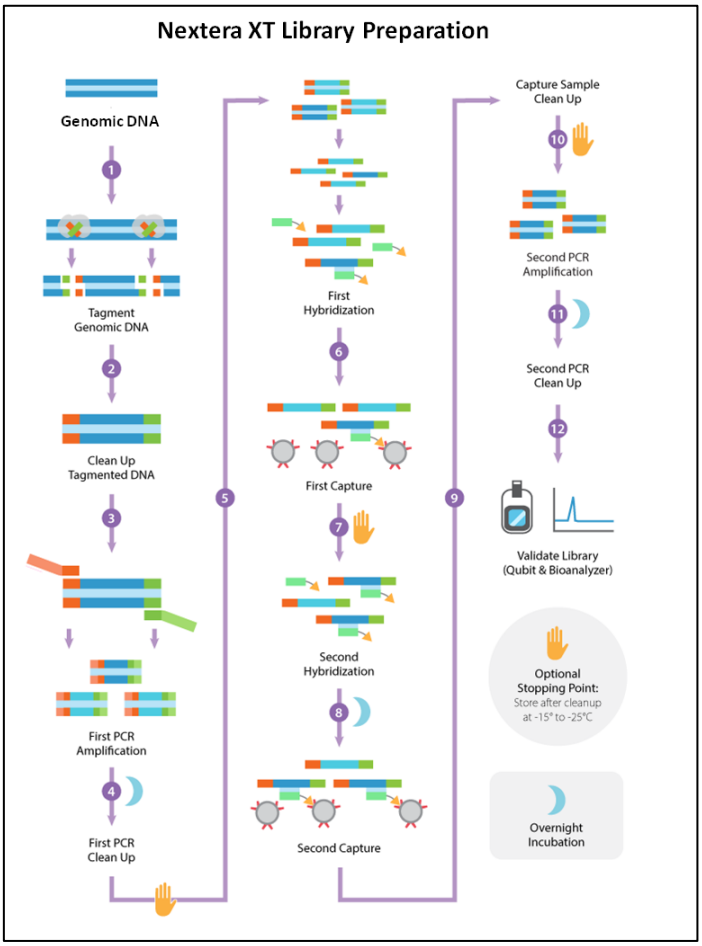


Figure 12:General flow chart for the Illumina Nextera XT DNA Library Prep kit

c) Exome sequencing:

Then study of the entire exome was carried out in a three generation family with four affected individuals, where OAVS segregated as Mendelian autosomal dominant trait. The exome analysis was performed by outsourcing with Ion Proton PCR based enrichment kit (Life Technologies). Libraries preparation was performed using the kit Ion AmpliSeq ™ exome RDY 1x8 Kit (Life Technologies). Starting from 100 ng of genomic DNA, libraries of 200 bp amplicons were generated. Each sample was indexed using adapters Ion Xpress Barcode 1-96. Adapter kit (Life Technologies) and then indexed amplicons were selected based on their length through the use of AmpureXP beads (Beckman Coulter). Finally, each library was quantified by qPCR using the KAPA Library Quantification Kits for IonTorrent platform (KapaBiosystems). Subsequently, an"emulsion PCR" was performed and the library was enriched by using respectively the Ion PI Template OT2 200 v2 kit and Ion One Touch 2 system with the Enrichment Facility ES (Life Technologies). The amplified libraries were loaded on PI ™ Chip v2 tool and sequenced with Ion PI 200 Sequencing Kit Kit v2. The minimum coverage obtained was 80X. The data obtained have been processed in our laboratory. The reads were aligned using the software Torrent Suite ™ (Life Technologies). The coverage of each library was obtained using the software Coverage Analysis Plugin (Life Technologies).

The variants calling was obtained throughout Variant Caller Plugin software applying the algorithm "Germ-Line High stringency" for the identification of (SNPs) and insertions / deletions (indels). For this purpose it was used a version of GATK (Genome Analysis Toolkit) optimized for the Ion Proton. The data analysis has allowed the identification of about 1 million variants that have been sorted considering the family segregation and evaluating the frequency of the prioritized variants in genetic consultation databases.

4. Results

In the present study, I have analysed a total of 84 OAVS patients (40 females and 44 males) with OAVS showing the minimal diagnostic criteria described by Tasse et al., 2005. Unfortunately, for 17 of them, no more clinical details were available. All patients presented ear anomalies including anotia/microtia (8%), preauricular pits or tags (61%), diplastic ears (72%), hearing loss (52%), stenosis of auditory canal (6%) and only one patients, who is a mother of two Goldenhar childrens with OAVS minimal criteria, showing exclusively asymmetric ear. Hemifacial microsomia was present in all of patients, mainly due to mandibular/malar hypoplasia (70%). Other features included eye and vertebral anomalies respectively present in 51% and 34% of patients. All clinical features were summarized in Table 3.

Table 3. Clinical features in our OAVS cohort

Clinical features	Patients (n)	Patients (%)
Total of patients	84	
Total of informative patients	67/84	80%
Gender F/M	30/37	45/55
Ear anomalies	84/84	
Anotia or microtia	5/67	8%
Preauricular pits or tags	41/67	61%
Dysplastic ear	45/67	72%
Hearing loss	35/67	52%
Asymmetric Ears	1/67	1,5%
Stenosis or Agenesia Auditory Canal	6/67	9%
Hemifacial microsomia	84/84	100%
Cleft lip/palate	14/67	21%
Facial nerve weakness	1/67	1,5%
Mandibular or malar hypoplasia	47/67	70%
Other craniofacial features	3/67	4,6%

Eye anomalies	34/67	51%
Epibulbar dermoids	17/67	25%
Coloboma	5/67	8%
Microphthalmia	16/67	25%
Other eye anomalies	9/67	13%
Vertebral anomalies	23/67	34%
Cervical spine malformations	18/67	27%
Dorsal spine malformations	6/67	9,2%
Esophageal atresia	5/67	8%
Pulmonary defects	1/67	1,5%
Congenital heart defects	18/67	27%
Genitourinary malformation	10/67	15%
Cerebral malformation	8/67	12%
Mental retardation	10/67	15%
Other organs/systems	7/67	11%

All 84 samples have been processed throughout different approaches (Targeted Sequencing,Nextera XT,Cytoscan HD) based on the quality and the available quantity of DNA.

SNP array analysis (Cytoscan HD, Affymetrix) was performed on 23 samples showing a wide phenotypic spectrum.

Only one sample was excluded from the data analysis because of the low quality of the data obtained. The data analysis has identified in 9 patients, 11 Copy Number Variant (CNVs) including 8 microdeletions and 3 microduplication, ranging from 80Kbp to 472 Kbp. Furthermore a large deletion of 5Mb was detected in a sporadic patient (see Table 4). For 7 patients was possible to evaluate the segregation of CNVs identified, showing that 4 CNVs were maternally inherited, 3 CNVs were paternally inherited and a deletion of 5Mb was found to have a *de novo* origin. These data contribute to enlarge the SNP array data obtained from the analysis of other 64 samples previously processed in our lab throughout SNP Chip 6.0 (Affymetrix) that will be argued in the next discussion section.

Table 4.CNVs results in 23 OAVS patients

SAMPLES	REARRANGEMENTS	GENES INCLUDED	INHERITANCE
26	arr[hg19] Xq28(148,622,779-148,780,308)x3_158Kb	*MAGEA11*	maternally inherited
55	arr[hg19] 12q23.1(99,839,854-99,939,825)x1_100kb	*ANKS1b*	unknown
	arr[hg19] 3q25.1(151,910,327-152,020,168)x1_110kb	*MBNL1*	
	arr[hg19] 1q31.3(198,581,014-198,661,358)x1_80Kb	*PTPRC*	
6	arr[hg19] 10q23.33(96,748,550-96,920,690)x1 _175Kb	*CYP2C9,CYP2C8*	unknown
4	arr[hg19] 13q21.33(71,995,356-72,230,384)x1_235Kb	*DACH1*	maternally inherited
50	arr[hg19] 8q23.3(113,436,510-113,671,918)x3_235K	*UNCD5*	maternally inherited
16	arr[hg19] Xp22.12(19,302,816-19,775,148)x3_472Kb	*PDHA1, MAP3K15, SH3KBP1*	paternally inherited
74	arr[hg19]16p13.13(12,065,052-12,227,061)X1 162 Kb	*SNX29*	paternally inherited
75	arr [hg19] 2q23.1(148,955,661-149,020,865)x1	*MBD5*	paternally inherited
68	arr[hg19] 15q26.2q26.3(97,018,658-102,429,112)x1_5410Kb	*IGFR1,ALDH1A3,MEF2A*	*de novo*
	arr[hg19] 9p22.1(18,853,814-18,973,795)x1_120Kb	*ADAMTSL1,FAM154A*	maternally inherited
62	Negative		
63	Negative		
15	Negative		
10	Negative		
49	Negative		
23	Negative		
56	Negative		
57	Negative		
58	Negative		
59	Negative		
12	Negative		
61	Negative		
9	Negative		

Targeted sequencing of 78 genes was performed in 65 samples (35 females; 30 males) throughout Truseq Custom Amplicon's panel. I have obtained an average coverage of about 700X and an expected 5% of regions with Q-value <30, which included the intronic regions flanking the coding portions of the target sequence. The data analysis have identified an average of 4 variants for each sample subsequently prioritized taking into account 12 different tools of pathogenicity such as SIFT, Polyphen, Mutation Assessor e CADD. At least, a

total of 31 genetic variants have been identified in 27 different samples, meanwhile 38 samples were negative (see Table 5). Segregation analysis was available only for 16 samples identifying a total of 19 variants, in detail 7 were paternally inherited, 10 were maternally inherited and 2 were *de novo* ones.

Segregation analysis was not available for other 12 variants detected in 11 samples.

All these variants involve 22 different genes (see Table 5) responsible for development of the first and second branchial arch, involved in intracellular transduction of many extracellular signals, implicated in vasculogenesis and essential for the normal functioning and cellular survival. 12 variants were identified in 6 genes involved in several diseases with overlapping clinical phenotype.

Table 5 Targeted sequencing results in 65 OAVS patients

Samples	Gender	Targeted sequencing results	Inheritance
1	M	CACNA1C: NM_001129840:[c.758-5C>T]	undetermined
2	F	CACNA1C:NM_001129837: [c.C5174G:p.A1725G]	paternally inherited
3	F	CHD7:NM_017780: [c.C5858T:p.A1953V]	maternally inherited,
4	M	CHD7:NM_017780: [c.A8266T:p.T2756S]	undetermined
5	M	CHD7:NM_017780: [c.A8477G:p.N2826S]	maternally inherited,
		NCOR1:NM_001190438:[c.C1704A:p.N568K]	maternally inherited,
6	F	DCN:NM_133503: [c.A424G:p.K142E]	undetermined
7	M	EGFR:NM_005228: [c.C2884T:p.R962C]	undetermined
8	M	EYA2 NM_005244:[c.979-7T>C]	*de novo*
9	M	FGFR2:NM_000141: [c.G1196C:p.R399P]	undetermined
10	M	GSC:NM_173849: [c.A161C:p.Y54S]	undetermined
11	M	HAND2:NM_021973: [c.C269G:p.P90R]	paternally inherited
12	M	LNPEP:NM_175920: [c.G113A:p.R38Q]	paternally inherited
		EFTUD2_ NM_004247.3:[IVS1058+3_1058+7het_delAAGTA]	maternally inherited,
13	F	NCOR1:NM_001190440: [c.T6343C:p.S2115P]	maternally inherited
14	F	MYOD1_NM_002478: [c.630+9G]	maternally inherited
15	F	NKX3-2:NM_001189: [c.G958C:p.G320R]	maternally inherited
		XXYLT1:NM_152531: [c.T311C:p.M104T]	paternally inherited
16	F	PLCB4:NM_001172646.1: [c.G2192A,p.G731D]	*de novo*
17	M	PLCB4:NM_182797: [c.1510+3G>A]	paternally inherited
18	M	POLR1C:NM_203290: [c.A193G:p.M65V]	undetermined

		SMAD4:NM_005359: [c.A1106G:p.N369S]	undetermined
19	M	POLR1C:NM_203290: [c.G490A:p.V164M]	maternally inherited
20	M	PUF60:NM_001271098: [c. C11G:p.A4G]	undetermined
21	M	SALL1:NM_002968: [c.A762G:p.I254M]	maternally inherited
22	M	SALL1:NM_002968: [c.G2713T:p.V905L]	paternally inherited
23	M	SEMA3E:NM_012431: [c.C2315T:p.T772M]	undetermined
24	F	SEMA3E:NM_012431: [c.C705A:p.D235E]	undetermined
25	F	LHX6:NM_001242335: [c.C457A:p.P153T]	Undetermined
26	F	SMAD4:NM_005359: [c.424+5G>A]	maternally inherited
27	M	SNX29:NM_032167: [c.1320-7T>C]	paternally inherited
28	F	Negative	
29	M	Negative	
30	M	negative	
31	F	negative	
32	M	negative	
33	F	negative	
34	F	negative	
35	M	negative	
36	F	negative	
37	F	negative	
38	M	negative	
39	F	negative	
40	M	negative	
41	M	negative	
42	F	negative	
43	M	negative	
44	F	negative	
45	M	negative	
46	F	negative	
47	F	negative	
48	F	negative	
49	M	negative	
50	M	negative	
51	M	negative	
52	F	negative	
53	F	negative	
54	M	negative	

55	M	negative	
56	F	negative	
57	M	negative	
58	F	negative	
59	F	negative	
60	M	negative	
61	F	negative	
62	M	negative	
63	F	negative	
64	M	negative	
65	F	negative	

More recently two missense mutation identified by WES approach in the *MYT1* gene (Lopez et al., 2016) prompted us to screen 73 available OAVS samples throughout Nextera XT DNA Library Preparation protocol (Illumina).

The sequence analysis of *MYT1* identified three new mutations in three different sporadic patients. A missense mutation p.(Glu289Lys) located in the exon 7, the other one p.(Thr782Ser) in the exon 14 and an inframe deletion c.917_919delAGG in exon 7 maternally inherited. All the variants have been confirmed by Sanger sequencing (see Table.6).

Table 6. *MYT1* mutations

Samples	*MYT1* mutations	dbSNP rs Number
42	NM_004535.2:c.917_919delAGG	rs147483668
10 bis	NM_004535.2:c.865G>A; p.(Glu289Lys)	rs141023903
56	NM_004535.2:c.2345C>G; p.(Thr782Ser)	rs78568430

Finally I have performed an exome analysis of a family of four affected individuals, where OAVS segregated as Mendelian autosomal dominant trait into two separate branches. The data analysis identified about 75000 variants.

These variants were selected and prioritized based on the familial segregation considering all the affected family members. This kind of analysis identified 3 variants (1 missense variation and 2 splicing variants) in 3 different genes (*ITGB4, RNF213, SHPRH*) (see Table.7). These variants confirmed by Sanger sequencing, were detected also in several related unaffected family members.

Table 7. Segregation of exome variants in affected and unaffected familiar members.

CASES	VARIANT 1	VARIANT 2	VARIANT 3
	ITGB4:NM_000213.3 c.5452C>G,p.Q1818E	RNF213: NM_001256071.2 IVS14922+3A>G	SHPRH:NM_173082.3 IVS4267-3T>C
F(II-III)	No	No	No
Case I	Yes	yes	Yes
Case II	Yes	yes	Yes
Case III	Yes	yes	Yes
F (IV)	No	No	No
M(IV)	Yes	Yes	Yes
Case IV	Yes	Yes	Yes
S (IV)	No	Yes	No
GF(IV)	Yes	Yes	Yes
GM(IV)	No	No	No
U(IV) /GS(I)	Yes	No	Yes
A(IV)/ GD(I)	No	No	Yes

Case I: mother of two OAVS children (Case II and Case III) with asymmetric ears ; Case IV: grandchild of Case I ; F:father ; M:mather ; S: sister; GF:grand father ; GM:grand mother ; GS:grandson ; GD: granddaughter ; U:uncle ; A: aunt.

5. Discussion

The development of high-resolution molecular techniques such as microarray has contributed significantly to the identification of microdeletions and microduplications leading to the identification of new contiguous gene syndromes and new disease genes implicated in the etiology of numerous congenital defects (Vissers et al., 2005 ; Potocki et al., 2007 ; Lupski., 2007; Wiszniewska et al., 2014). In this study, I have analyzed 23 patients with

OAVS phenotype, showing at least two of the OAVS minimum criteria described by Tasse et al., 2005, such as mandibular hypoplasia, ear anomalies and the presence of fistulae or preauricular tags. The analysis carried out throughout genome-wide Cytoscan HD array identified 11 microrearrangements including 3 microduplications, 8 microdeletions and a large *de novo* deletion (see Table 4). A previous study performed in our laboratory on 64 OAVS patients showed the presence of 26 CNVs consisting in 11 microdeletions and 15 microduplications ranging from 80 to 1Mb and including 4 *de novo* rearrangements (microdeletions 8q24.3 and 4q35.2; microduplications Xq21.1 and 3q29). The data produced in this study contribute to expand results obtained on an extended cohort of 64 OAVS patients leading the number of examined patients to a total 87 samples. In particular, I identified a new *de novo* deletion encompassing about 5 Mb (Figure 13) on chromosome 15q26.2-q26.3 in a patient (code number 68) showing a full-blown OAVS phenotype including growth retardation, mandibular and maxillar hypoplasia, microretrognathia, preauricular tags, corneal dermolipoma, cataract, vertebral and congenital cardiac anomalies (consisting in patent foramen ovale, patent ductus arteriosus, mild interventricular shunt, mild interventricular defect, slightly dilated hypertrophic right ventricle). Moreover, the patient's phenotype was complicated by simple tooth decay and slight learning delay. Patient had a normal thyroid profile and the bone age corresponded to chronological age.

Patient was tested as negative to the analysis of the *IGF1* gene. SNP-array analysis identified also a maternally inherited microdeletion covering 120 Kb on chromosome 9p22.1 and including *FAM154A* gene. *De novo* terminal deletions on 15q are rare (Roback et al., 1991 ; Pinson et al., 2005). To date, only 20 cases have been reported harboring pure distal 15q26 deletions (Roback et al., 1991 ; Pinson et al., 2005). Clinical features associated to distal 15q26 deletions usually include intrauterine and post-natal growth retardation, microcephaly, skeletal anomalies, facial and auricular dismorphisms, that include microcephaly, low set ear and shaped ear anomalies. Interestingly, mandibular and maxillar hypoplasia and preauricular pits or tags, which are the minimal features in OAVS individuals and were present in our patient, have not been described in patients with 15q26.2-q26.3 deletion. This region was also previously associated to OAVS in a linkage study performed on a three-generation family with Goldenhar syndrome. More in details, affected members

of this family presented with facial asymmetry and preauricular pits, ocular anomalies, including epibulbar dermoid and, in a single patient, cardiac and vertebral anomalies. The 15q26.2-q26.3 deletion identified in our cases involves 40 genes. Among these, two gene, namely *IGF1R* gene (insulin growth-factor receptor), which is involved in the intrauterine and post natal growth retardation and the *MEF2A* (myocyte enhancer factor A), which is involved in the development of central nervous and cardiovascular systems, could explain some of the clinical features of the patient.

15q26.2-q26.3 deletion also involves the *ALDH1A3* (aldehyde dheidrogenase 1 family member A3), an aldehyde dehydrogenase enzyme that uses retinal as a substrate during the early stages of embryonic development contributing to the formation of a retinoic acid (RA) gradient along the ventral and dorsal axis. The involvement of retinoic acid in the OAVS etiology has been previously demonstrated and recently, two mutations in *MYT1*, another gene involved in the RA pathway, were described in two patients with OAVS phenotype. Taken together, present and previous studies suggest the involvement of RA pathway in OAVS etiology (Lopez et al., 2016).

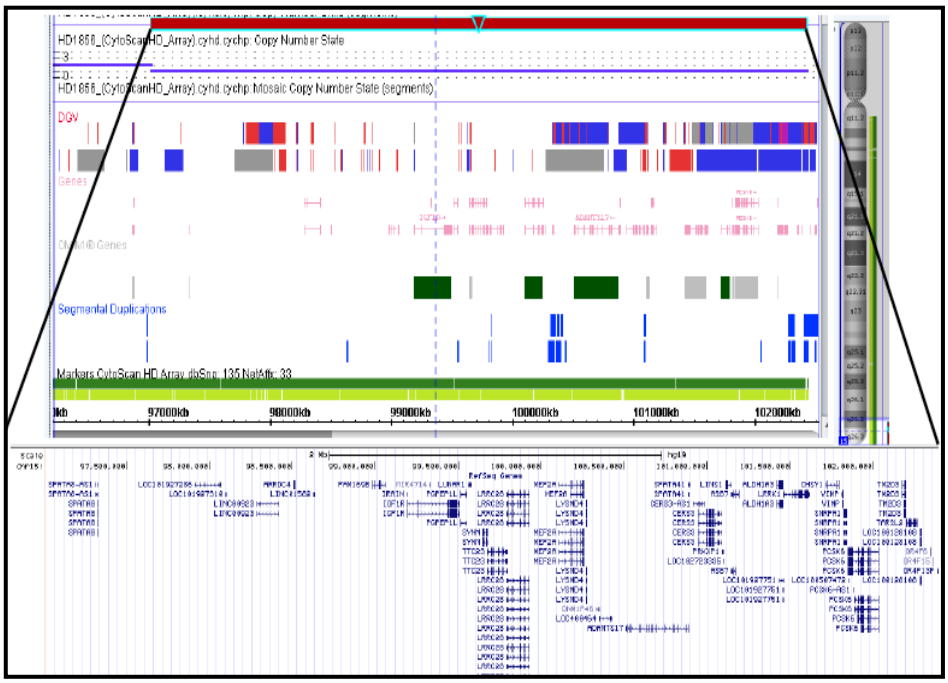


Figure 13. Rapresentation of the new *de novo* deletion encompassing about 5 Mb on chromosome 15q26.2-q26.3 involving 40 genes throughout Chromosome Analysis Suite (ChAS)

Another patient (code number 55), was found to be heterozygous for three different microdeletions located on chromosomes 12q23.1, 3q25.1, 1q31.3 of various size (ranging from 80 to 110 Kb). For none of these cases parental DNA was available to verify the deletion origin. None of these rearrangements was previously reported in patients with OAVS and the genes encompassed by these deletions were not functionally related to alterations of embryonic development. Therefore, the relationship of these deletions with disease remains uncertain, although their correlation with disease cannot be excluded since these microriarrangements may act synergistically as susceptibility variants contributing to the onset of the disease (Hehir-Kwa et al., 2013).

Seven patients had CNVs inherited from an healthy parent. However, incomplete penetrance and variable expression are described in OAVS as shown by a study in which retrospective analysis of families segregating OAVS showed that 45% of not affected OAVS parents or relatives had small signs of the disease, and therefore a contribution of these variant to disease expression cannot be excluded as well.

These microrearrangements include the *SNX29* gene identified in our previously SNP-array analysis and the *DACH1* gene involved in the RA pathway as well as the PAX, EYA and SIX pathway. In particular, the deletion including *DACH1* gene (13q21.33del) was identified in a patient (Patient 4) showing facial asymmetry, dysplastic ears, celebral malformation and developmental delay. This gene encodes for a transcription factor homologue to the Drosophila *dachshund* gene, which is a key regulator of cell fate determination during eye, leg and brain development in the fly (Heanue et al., 2002). *Dach1*, as well as its paralog *Dach2*, are excellent candidates for OAVS because they are expressed during the early stage of embryonic development and correlate with Pax, Eya, and Six pathway, essential for proper development of the pharyngeal arches (Heanue et al., 2002). Another rearrangement, a *de novo* duplication of 75 Kb including the *DACH2* gene, a paralog of *Dach1*, was identified in a patient previously analyzed by SNP-array. In addition to the peculiar characteristics of the OAVS phenotype, both patients exhibited cardiac defects involving the aortic outflow tract and in particular, the presence of an

anomalous pulmonary venous return. Remarkably, mutations in *EYA1*, *SIX1* and *SIX5* genes have been described in patients with BOR syndrome, a condition characterized by abnormalities of the pharyngeal arches (clefts, fistulae, cysts), hearing defects and kidney abnormalities. The presence in BOR syndrome of phenotypic characteristics partially overlapping with OAVS led some authors to consider this condition as the most severe expression of the phenotype associated with OAVS. Involvement of the EYA, PAX, SIX pathway in OAVS was further suggested by the recent identification of a duplication on chromosome 14q23.1 region in a family with autosomal dominant OAVS, including the *OTX2* gene, (Ballesta-Martinez et al., 2013). Of note, this duplication (14q22.3-q23.3), in addition to the *OTX2* gene, included *SIX1* and *SIX6*. Consistently with the phenotype associated to mutation of these genes, the affected patients of this family (a father and his son) presented with features overlapping both OAVS and BOR syndrome (Ou et al., 2008). More interestingly, the *EYA*, *PAX*, *SIX* pathway is also involved in the Townes-Brocks syndrome, another condition phenotypically related to OAVS and caused by mutations in *SALL1* gene. Indeed, alterations in the *SIX1*, *SIX5* and *EYA1*, which acts as a cofactor within the pathway, lead to reduced expression of *SALL1*, altering both the end of the program by setting *HOXA2* expression, and reducing the overall expression of the entire regulatory network controlling the formation of branchial arches derivatives (Cox et al., 2014).

Another interesting case characterized in this thesis, is patient 6 showing facial asymmetry, preauricolar pits and tags, dysplastic ears and a mild mental delay. This patient carries a 10q23.33 microdeletion extending over 175 Kb including two genes, *CYP2C9* and *CYP2C8*, encoding for two of the four members of the CYP2C family (Gelboin and Krausz., 2006 ; Gerbal-Chaloin et al., 2001). These genes are interesting since one of the pathophysiological mechanisms hypothesized for OAVS involves the alterations of vascular processes. Indeed, *CYP2C8* and *CYP2C9* are expressed in human endothelial cells (ECs) (Hillig et al., 2003 ; Lin et al., 1996), where they metabolize endogenous arachidonic acid into vasoreactive epoxyeicosatrienoic acids (EETs). Furthermore, CYP2C8/9-derived EETs are involved in the process of angiogenesis under hypoxia (Sullivan-Klose et al.,1996 ; Sim et al., 2006). In an angiogenesis model *in vitro*, the EET antagonist inhibited tube formation induced by *CYP2C9* overexpression, suggesting that CYP2C-derived EETs

significantly affect the sequence of angiogenic events under hypoxia (Sullivan-Klose et al., 1996). Angiogenesis is the formation of new blood vessels, and vascular endothelial growth factor (*VEGF*) plays a central role in this step. The *VEGF* gene is enhanced under hypoxic conditions and controlled primarily by hypoxia-inducible factor-1 (HIF-1) (Limdi et al., 2008b). HIF-1 is composed of HIF-1α and Arnt(aryl hydrocarbon receptor nuclear translocator) (Limdi et al., 2008a). Hypoxia induces its expression by stabilizing the hypoxia inducible factor-1 (HIF-1), which binds to the hypoxia response element (HRE) within the *VEGF* promoter and strongly enhances its transcription. *VEGF* was recently reported to activate the promoter of *CYP2C9* and enhance the expression of *CYP2C8* mRNA and protein in endothelial cells (Webler et., 2008). This observation imply that alternative pathogenetic mechanisms occur in these families, including the occurrence of different genetic alterations, such as point mutations or small deletion and/or insertion, which by definition excape SNP-array analysis, or epigenetic alterations, which are have been hypothesized in the OAVS condition based on the observation on tweens and in pregnancies obtained with in vitro fertilization. SNP-array analysis of further cases contributed to expand the number of individuals in our OAVS cohort for which SNP-array data are available. In total, we have now analyzed 87 patients and identified 37 CNVs, five of which where demonstrated to be due to *de novo* events. Consistently with observations performed on other sindromes, the number of microduplications overtook the number of deletions also in OAVS. This result could be explained by the fact that duplications are generally better tolerated than deletions in human genome (Lindsley et al., 1972 ; Shaffer and Lupski., 2000). Haploinsufficiency is, in fact, most frequently associated with phenotypic abnormalities, while an overdose of gene content, in most cases, has less serious consequences (Redon et al., 2006 ; Conrad et al., 2006 ; McCarroll et al., 2006 ; Khaja et al., 2006). Nevertheless, as demonstrated for numerous other syndromes linked to chromosomal duplications, like duplication 17p12 which is associated to peripheral neuropathy and Charcot-Marie-Tooth disease (Potocki et al., 2007 ; Lupsky et al., 2007), the presence in these rearrangements of genes susceptible to dose-effect, or the breaking of genes by chromosomal breakpoints could provide an explanation for the pathogenic mechanism associated to the duplication. Although no recurrent deletion or duplication was evidenced in our study, the identification of multiple rearrangements including

de novo events and transmitted deletions and duplications encompassing genes functionally related to cranial structures development, emphasize the importance of applying SNP-array analysis to the genetic characterization of the OAVS condition. At the same time, the identification of multiple events leading to the same OAVS phenotype reinforce the hypothesis that OAVS represent an heterogenic condition. OAVS is the paradigmatic condition refractory to traditional gene discovery approaches for several reasons. Indeed, locus heterogeneity, the availability of small cohorts, the absence of large multi-generational families, had made difficult to attack OAVS using traditional gene discovery approaches. The advent of next-generation sequencing (NGS) surmounts these issues and changes the landscape of rare-genetic-disease research, with the possibility of identifying genetic disease causative genes at an accelerating time-rate.

Starting from the genes enclosed in the rearrangements identified by SNP-array, and using several bioinformatics software tools like Biogrid, GeneMania, String, and IPA, that allow to highlight the correlations and interactions present between genes, we generated an OAVS candidate network including genes implicated in the development of the first and second branchial arches and genes related to disorders with phenotypes partially overlapping with OAVS. The OAVS candidate gene panel included 78 genes. This list of genes was used to build a NGS OAVS targeted panel which was applied to the analysis of 65 patients. After filtering and prioritization, NGS analysis identified a total of 31 genetic variants in 27 independent samples, 12 of which involving genes related to disorders with clinical features overlapping OAVS.

In particular, the novel missense variant p.(Tyr54Ser) was found in the *GSC* gene in a patient (code number 10) showing microtia, preauricolar pits and tags, facial asymmetry, mandibular hypoplasia and macrostomia. The *GSC* gene encodes for the Goosecoid Homeobox, a homeodomain-containing protein present in many species and implicated in a variety of key developmental processes. Originally reported as a gene involved in organizing the embryo during early development, *GSC* was demonstrated to be expressed during organogenesis, most notably in the head, limbs and ventrolateral body wall. Genetic analyses in mice provided evidence that numerous homeobox genes, including *Msx*, *Dlx*, *Gsc* and *Prx* as well as other transcription factors such as *Hand2*, are expressed in pharyngeal arch mesenchyme and plays an essential

role in development of the first pharyngeal arch. To investigate the role of *GSC* in embryonic development, Yamada et al. (1995) inactivated the gene by gene targeting to generate a mutant mice. Mutant homozygous mice for the *Goosecoid* mutation do not display a gastrulation phenotype and are born; however, they do not survive more than 24 hours. Phenotype analysis of the homozygotes mice revealed numerous developmental defects affecting those structures in which *GSC* gene is expressed during its second (late) phase of embryonic expression. Predominantly, these defects involve the lower mandible and its associated musculature including the tongue, the nasal cavity and the nasal pits, as well as the components of the inner ear (malleus, tympanic ring) and the external auditory meatus. A genome wide search for linkage in two families with features of HFM was performed to identify the disease loci. In one family data were highly suggestive of linkage to a region of approximately 10.7 cM on chromosome 14q32, with a maximum multipoint lod score of 3.00 between microsatellite markers D14S987 and D14S65. This locus harbours the *Goosecoid* gene. No pathogenic genomic variant was detected analyzing the coding regions of *GSC* in the two HFM families and in 120 sporadic cases. The phenotype of affected individuals in this family was highly variable ranging from mild to more severe form, including macrostomia, bilateral preauricular skin tags, cleft palate , narrow external auditory meati, some speech delay, micrognathia, tracheo-oesophageal fistula and oesophageal atresia and mild or marked facial asymmetry (Kelberman et al., 2001).

The missense p.(Tyr54Ser) variant identified in our patient, which was predicted by bioinformatics tools to be deleterious/desease causing, involves the first of the three exons of *GSC* gene, leading to a change of the highly conserved Tyr^{54} amino acid residue (considering 11 species, Tyr^{54} is conserved up to Frog) to Ser, a missense substitution exchanging two amino acids with large physicochemical differences(Figure 14) suggesting a pathogenic mechanism potentially involving *GSC* protein function as well as its interaction with other molecular interpreters belonging to the functional genetic network underlyng first and second branchial arch development. Such a mechanism, could explain the craniofacial anomalies showed by our patient. Animal studies will be essencial in order to correlate the novel mutation p.(Tyr54Ser) to phenotipical characterization of mutated patient. To our knowledge, considering

the OAVS cases with normal karyotypes so far described into literature, p.(Tyr54Ser) is the first missense variant identified in the *GSC* gene.

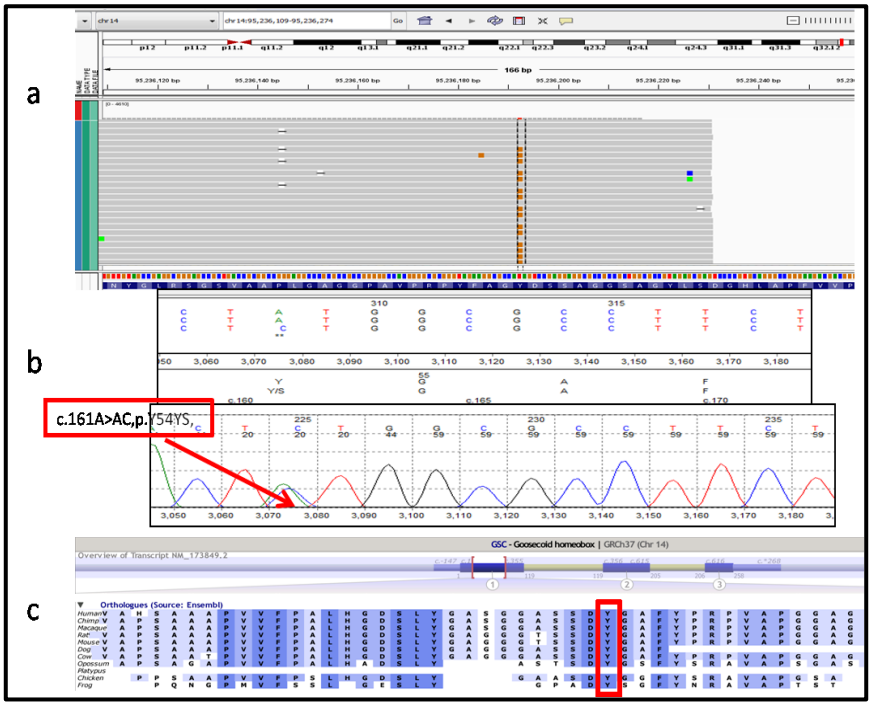


Figure 14.a) The Integrative Genomics Viewer (IGV**),** a high-performance visualization tool for interactive exploration of next-generation sequence data, allowing to verify the coverage of the variants; b) Mutation surveyor software for DNA Sanger sequence analysis ; c) Alamut software suite, a set of tools designed to help analyze and interpret mutations in human genetics allowing to evaluate the specific conservation of the amminoacidic residue interested by the mutation.

Analysis identified another interesting variant, a de novo proven missense change p.(Gly731Asp), in the *PLCB4* gene. The patient harboring the variant (patient 16) had facial asymmetry, cleft palate, mandibular hypoplasia, pulmonary defects, and congenital heart disease (scimitar syndrome). This gene is relevant to OAVS since *PLCB4* belongs to a group of enzymes (phospholipases) that hydrolyze phospholipids into fatty acids and other lipophilic molecules, and it is predicted to function as a signaling molecule

whithin the endothelin 1 (EDN1)–endothelin receptor type A (EDNRA) pathway, which is important for patterning of the pharyngeal arches in animal models (Uuspää., 1978 ; Storm et al., 2005). Missense mutations in *PLCB4* were reported to be associated with auriculocondylar syndrome (ACS; MIM 602483; also known as "question-mark ear syndrome" or "dysgnathia complex") an autosomal-dominant craniofacial syndrome characterized by highly variable mandibular anomalies ranging from mild to severe micrognathia, often with temporomandibular joint ankylosis, cleft palate, and a distinctive ear malformation, that consists of separation of the lobule from the external ear, giving the peculiar appearance of a question mark (Rieder et al., 2012).

Moreover, whitin the ACS literature, postauricular tags have been reported in a small number of cases (Storm et al., 2005 ; Masotti et al., 2008 ; Gerkes et al., 2008 ; Hunter et l., 2009). In particular , in a recent study Gordon et al., described one case showing a postauricular tag in addition to the canonical features of ACS, carrying a mutation at Arg621 or a homozygous deletion within *PLCB4*(Gordon et al., 2013). Differently from the missense variant Arg621, our missense variant, p.(Gly731Asp), involves two different functional domains C2 calcium-dependent membrane targeting and C2 membrane targeting protein suggesting the potential alteration of some binding sites and the affection of the subsequent signal transduction.

It has been suggested that the auricular phenotype in ACS patients, all showing the typical" question mark ear",varies widely, and a recent report expanded the spectrum of ACS-associated anomalies to include ear phenotypes that might otherwise fall within OAVS/GS or other branchial arch syndromes(McGowan et al., 2011). Our patient also presented with some milder phenotypical features partially overlapping with ACS such as cleft palate and mandybular anomalies, but did not show the distinctive features of ACS like question mark ears.

Considering *PLCB4* gene as one of the molecular interpreters of the first and second branchial arch developmental pattern and that our patient lack of the distinctive sign "question mark ear", the missense mutation p.(Gly731Asp) in *PLCB4* gene could be considered as an OAVS-related allele, contributing or causing a phenotype presenting features partially overlapping with ACS.

We also identified three patients (number 3, 4 and 5) harbouring three separate missense variants in *CHD7* (Chromodomain Helicase DNA Binding Protein 7) gene, namely p.(Ala1953Val), p.(Asn2826Ser), and p.(Thr2756Ser). CHD7 belongs to a family of highly conserved proteins involved in the regulation of transcription and plays a significant role in early embryonic development controlling gene expression through its activity in chromatin remodeling . *CHD7* gene is mutated in CHARGE syndrome. In particular, nonsense and frameshift variants with predicted protein truncation and nonsense mediated decay are typically considered causative of CHARGE syndrome whereas, *CHD7* missense variants are not considered pathogenic unless reported to be *de novo*. In our cases, parental DNA was available only for patients 3 and 5, and in both cases segregation analysis revealed maternal transmission of the missense variant [p.(Ala1953Val) and p.(Asn2826Ser)].

Despite both variants were transmitted by an apparently unaffected parent, both variants had a frequency lower than 0.001% in the general population's databases available online, like dbSNP 149 (https://www.ncbi.nlm.nih.gov/projects/SNP/), GO-ESP (http://evs.gs.washington.edu/EVS/) and ExAC (http://exac.broadinstitute.org/). Moreover, they were both predicted as deleterious by five pathogenicity predictors, *e.g.*, SIFT, PolyPhen2, MutationAssessor and CADD. Therefore, involvement of these variants in the OAVS phenotype observed in our patients cannot be excluded. Indeed, consistent with a CHD7-related phenotype, patients 3, 4 and 5 clinical presentation included phenotypic features overlapping with CHARGE syndrome like facial nerve weakness, congenital cardiac abnormalities, cerebral malformations and developmental delay, although the presence in these patients of OAVS typical features such as epibulbar dermoid and microftalmia allow us to include these patients in the OAVS.

NGS analysis detected two distinct missense changes [p.(Thr772Met) and p.(Asp235Glu) missense variant] in the *SEMA3E* gene, encoding for semaphorine 3E in two independent patients (numbered 23 and 24). Interestingly, also this gene has been linked to CHARGE disease. In particular a *SEMA3E de novo* missense variant was found in a patient with retinal coloboma, choanal atresia, ear malformation, hearing loss, facial palsy, atrial septal defect, and growth retardation (Lalani et al., 2004). Patient 23 had the

minimum diagnostic OAVS criteria (Tasse et al., 2005), but no additional clinical information was available, whereas patient 24 showed microtia, dysplastic ears, hearing loss, facial asymmetry and mandibular hypoplasia. For both of them, parental DNA was unavailable and was therefore impossible to evaluate if the variant was *de novo* or transmitted. However, both variants were found to be rare in the general population based on consultation of distinct databases. P.(Thr772Met), identified in patient 24, was considered as a polymorphic varinant by use of several pathogenic predictors, whereas the same bioinformatics tools predicted p.(Asp235Glu) to be a desease causing allele. Consistent with a pathogenic role of *SEMA3E* p.(Asp235Glu), this variant is located in the Semaphorin/CD100 antigen domain including the SEMA3E-PlexinD1 binding site with functional protein significance. Interestingly, SEMA3E-PlexinD1 signaling plays an essential role in vessels regeneration as well as rewiring of synapses, and vascular impairment was originally proposed as one of the possible mechnaisms underlying OAVS. Despite the clinical presentation of our *SEMA3E*-mutated OAVS patients was different from the CHARGE-like *SEMA3E* mutated patient described in literature (Lalani et al., 2004), the identification of many variants with likely pathogenetic significance in genes that are frankly related to syndromes with phenotypical overlap to OAVS, suggests that current mutated genes are involved in OAVS as well, possibly contributing to disease through an allelic series or through low penetrant alleles that in combination with other, generate the phenotype.

Remarkably, we identified a missense variant [p.(Met104Thr)] in *XXYLT1*, a gene that we previously found to be deleted in a patient with OAVS that we described in a previous work. This patient (patient 15) presented with dysplastic ears, hearing loss, facial asymmetry, mandibular hypoplasia, cervical and dorsal spine malformations. This missense mutation was found to co- segregate with another missense variant, the p.(Gly320Arg) allele in the *NKX3-2* gene. *XXYLT1* p.(Met104Thr) was paternally transmitted whereas *NKX3-2* p.(Gly320Arg) was transmitted by the mother. The microduplication involving *XXYLT1* gene previously identified in our laboratory is a 729 Kb rearrangement found in a patient with an OAVS form characterized by preauricolar pits and tags, dysplastic ears, hearing loss, facial asymmetry, mandibular hypoplasia and microphtalmia (Guida et al., 2014). *XXYLT1* gene encodes for an endoplasmic reticulum localized xylosiltransferase, which can transfer a second xylose to

different epidermal growth factor (EGF)-like domains of the Notch protein (Sethi et al., 2012). Of note, *drosophila* models with decreased Notch xylosylation indicate that xylose residues on EGF16–20 negatively regulate the surface expression of the Notch receptor (Lee et al., 2013), which is an interesting observation considering that animal studies showed that Notch genes are expressed during the development of the inner ear, driving the sensory program in nonsensory cells (Pan et al., 2013), and play an important role during vascular morphogenesis (Roca and Adams., 2007). More interestingly, the other putative contributing gene, *NKX3-2* (NK3 Homeobox 2), also known as *BAPX1* gene, encodes for a member of the NK family of homeobox-containing protein, involved in skeletal development. Remarkably, a previously mutation analysis of *BAPX1* on 105 OAVS samples failed to identify genic mutations, but found some evidences of epigenetic dysregulation of this gene in OAVS (Fisher et al., 2006). In addition, involvement of this gene in skeletal morphogenesis was supported by a *Nkx3.2* mutant mice that died perinatally because of a skeletal phenotype characterized by a reduction of the ventro-medial parts of vertebral bodies, most prominent at the level of cervical vertebrae (Akazawa et al., 2000 ; Lettice et al., 2001 ; Tribioli and Lufkin., 1999).

The *XXYLT1* variant was predicted as disease causing by two of five predictors of mutation pathogenicity, whereas the *BAPX1* was predicted to be disease-related in all prediction analyses. Consitent with a prominent role for *NKX3-2* p.(Gly320Arg) variant respect to *XXYLT1* p.(Met104Thr) variant, *NKX3-2* Gly^{320} amino acid is an higly conserved residue, whereas *XXYLT1* $p.Met^{104}$ is less conserved. It could be hypothesized that the two variants contribute to the OAVS phenotype both behaving like recessive alleles when present alone but acting in an additive or synergistic fashion when both present in the same patient (double heterozygote). In another patient , we found a novel missense variant p.(Ala4Gly) in the *PUF60* gene. This variant was identified in a patient (numbered 20) showing microtia, preauricolar pits and tags, facial asymmetry, cleft palate, mandibular hypoplasia, microphtalmia, cervical spine malformation, esophageal atresia, congenital heart defects (atrial and ventricular septal defects) and celebral malformations. Remarkably, *PUF60* gene is located in 8q24.3 region, which was found to be involved in a *de novo* deletion encompassing 0.1 Mb in a patient with peduncles and preauricular

fistulas, mild facial asymmetry, patent ductus arteriosus and growth retardation. *PUF60* (Poly(U) Binding Splicing Factor 60) encodes a nucleic acid-binding protein that plays a role in a variety of nuclear processes, including pre-mRNA splicing and transcriptional regulation. The loss of function for Puf60 and Scrib genes in the *zebrafish* was reported to cause microcephaly and craniofacial defects. Moreover, *in vitro* experiments showed that the simultaneous reduction of the expression of *Puf60* and *Scrib* genes correlates significantly with the short stature also present in the patient (Dauber et al., 2013). Thus, the identification of the p.(Ala4Gly) variant in an OAVS patient corroborates the involvement of this gene in the etiology of OAVS. Moreover, the absence in our patient of short stature, together with the presence of the PUF60 variant, suggests that *SCRIB* gene might be involved in growth retardation.

The recent identification of two deleterious mutations in the *MYT1* gene in two unrelated patients with OAVS throughout WES approach in a study of 2016 carried out by Lopez et al., reporting Myelin transcription factor 1 (*MYT1*) as a new candidate gene for OAVS, brought us to screen 73 OAVS patiens in order to further confirm or exclude *MYT1* mutations in/from our cohort.

MYT1 (or neural zinc finger 2, *NZF2*) encodes the poorly characterised *MYT1* belonging to a non-classical CC-type zinc finger family of three proteins, including *MYT1L* neural zinc finger 1 (*NZF1*) and *ST18* neural zinc finger 3 (*NZF3*). All three genes are predominantly expressed in developing mammalian neural tissues (Kim et al., 1997). *MYT1* is part of the lysine-specific demethylase 1 complex that plays a crucial role in the neural cell-specific regulation of gene expression (Yokoyama et al., 2014). *MYT1* may also regulate pancreatic islet cell development (Gu et al., 2004) and its inactivation induced glucose intolerance in mice (Wang et al., 2007) potentially evoking maternal diabetes, a suspected cause of OAVS features in human fetuses.

Interestingly, *MYT1* belongs to RA-induced transcriptome as shown in inner ear development of mesodermal-knock-out Tbx1 conditional mouse mutants (Monks and Morrow., 2012) and in zebrafish early embryogenesis experiments.

In X. laevis embryos, RA treatment expanded the expression domains of several positive regulators of neurogenesis such as myt1, nrgn1 and gli3 and directed the neural plate towards a uniform proneural territory (Franco et al., 1999). *MYT1* was also shown to specifically interact with the RA response

element that is the cis-regulatory element present in the promoters of RAR-regulated genes(Gamsjaeger et al., 2013). Interestingly, depletion of RARs led to unbalanced RA signalling pathway and could result in developmental defects.

I have identified three variants in *MYT1* gene in three sporadic patients: Two missense variants p.(Glu289Lys) and p.(Thr782Ser) and an inframe deletion (c.917_919delAGG) rspectively in patients 10bis, 56 and 42. All three patients presented with the minimum diagnostic criteria described by Tasse et al., 2005, but no other clinical details were available. Segregation analys was carried out only for patient 42 revealing that the inframe detection identified was inherited from the healty mother. Both inframe deletion as well as p.(Glu289Lys) missense mutation were found to be rare in the general population based on consultation of distinct databases, meanwhile the p.(Thr782Ser) had a frequency of about 1% in the general population's databases available online. Despite that, p.(Thr782Ser), missense mutation identified in patient 10bis, was considered as a desease causing variant by use of several pathogenic predictors, whereas the same bioinformatics tools predicted p.(Glu289Lys) to be tolerated.

In addition, segregation of our inframe deletion (c.917_919delAGG) and the missense mutation identified by lopez et al., from an healthy non OAVS parent, could also confirm an incomplete penetrance mechanism underlying OAVS. Furthermore, the previously reported mutations associated with OAVS phenotype and the involvement of *MYT1* gene in the retinoic acid pathway confirm that this gene is causative of OAVS. Further investigation as well as studies in animal models may be helpful in order to better understand the potential pathogenecity of the *MYT1* mutations identified in our patients.

In a subsequent study, we analyzed by whole exome sequencing a family with four affected individuals, in which OAVS segregated as a Mendelian autosomal dominant trait with incomplete penetrance in two separate family branches (Figure15).

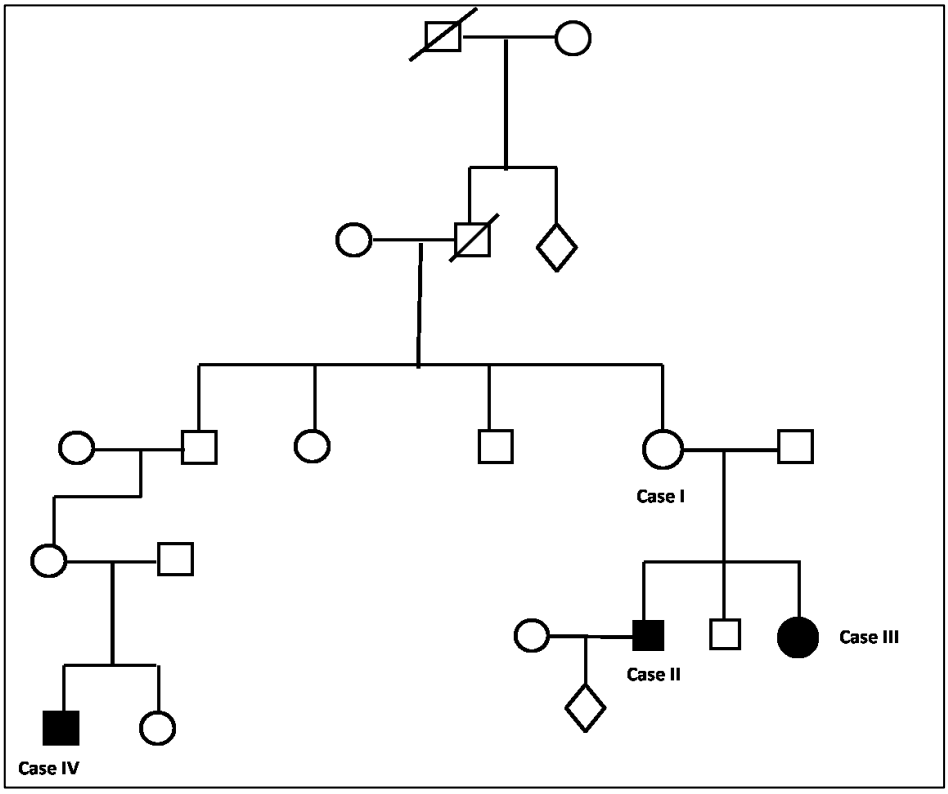


Figure 15. Example of family where OAVS segregated as a Mendelian autosomal dominant trait with incomplete penetrance in two separate family.Case I(mother of Case II and III)_Female patient with isolated asymmetry of the ears. Case II_ male patient showing facial asymmetry and agenesis of the right ear .Case III_famale patient with facial asymmetry, microtia and atresia of the right auditory canal, mixed hearing loss associated with slight sensorineural deficit on high frequencies on the left ear .Case VI_ grandchild of case 1, presented with a "full-blown Goldenhar syndrome" phenotype with facial asymmetry, epibulbar dermoid, monolateral otodisplasia , bilateral hearing loss, cervical spine malformation, agenesis and dislocation of the right kidney in the pelvic, radial involvement with the thumb agenesis and angled position of the metacarpals respect to the longitudinal axis of the ulna, and absence of the radio.

The family was found to be negative for CNVs and point mutations by SNP-array analysis and targeted NGS sequencing. The family shows an heterogeneous phenotype presentation characterized in one of the branch, consisting of a mother with two OAVS children, by isolated asymmetry of the ears (observed in the mother, case 1), facial asymmetry and agenesis of the right ear (observed in the eldest son, case 2), and facial asymmetry, microtia and atresia of the right auditory canal, mixed hearing loss associated with slight

sensorineural deficit on high frequencies on the left ear (observed in the female daughter, case 3). In the second branch of the family, the grandchild of case 1, presented with a "full-blown Goldenhar syndrome" phenotype with facial asymmetry, epibulbar dermoid, monolateral otodisplasia , bilateral hearing loss, cervical spine malformation, agenesis and dislocation of the right kidney in the pelvic, radial involvement with the thumb agenesis and angled position of the metacarpals respect to the longitudinal axis of the ulna, and absence of the radio (case 4). After filtering and prioritization, NGS analysis disclosed a total of 42 nucleotide variants. Filtering was performed by selecting all exonic and splicing variants with a frequency < 1% in several population databases. Further filtering and prioritization based on familial segregation and on variation frequency based on an internal variation database reduced the number of candidate variants to three, a missense change and two splicing variants shared by all the affected members of the family. The splicing variant IVS14922+3A>G was found in the *RNF213* gene (NM_001256071.2) encoding for a protein containing a C3HC4-type RING finger domain, which is a specialized type of zinc-finger domain. The *RNF213* protein also contains two regions called AAA+ ATPase domains that typically regulate mechanical processes in the cell, such as protein unfolding, DNA unwinding, or transporting molecules. Although the function of the RNF213 protein is undetermined, some studies suggest that it plays a role in the proper development of blood vessels (Fujimura et al., 2014 ; Liu et al., 2011). Indeed mutations in the *RNF213* gene have been associated with Moya-moya disease (MMD), a cerebrovascular disorder characterized by occlusive lesions of the circle of Willis. Allelic variations in *RNF213* are known to confer the risk of MMD by the down regulation of matrix metalloprotease in endothelial cells. As previously stated, vascular formation impairment has been proposed as the possible mechanism for pathogenesis for a number of defects, including transverse-limb defects, oromandibular-limb hypogenesis sequence, in addition to OAVS. Another splice variant, the c.IVS4267-3T>C, was found in the *SHPRH* (NM_173082.3) (acronym for *SNF2*, histone linker, PHD, RING, helicase) gene, a E3 ubiquitin-protein ligase that contains motifs characteristics of several DNA repair proteins, transcription factors, and helicases. In particular, mutations affecting the PHD, H15 linker and the RING finger domains identified in other genes such as *ATRX*, *MORF* or *BRCA1*, indicate a

role in regulation of gene expression via DNA methylation suggesting that *SHPRH* plays a role in chromatin-mediated transcriptional regulation.

Finally, we have found the missense variant c.5452C>G in the *ITGB4* (NM_000213.4) (Integrin alpha-6/beta-4) gene, a laminin-5 receptor predominantly expressed in squamous epithelial cells, endothelial cells, immature thymocytes, Schwann cells, and fibroblasts of the peripheral nervous system. *ITGB4* plays a critical role in the hemidesmosome of epithelial cells and is required for the regulation of keratinocyte polarity and motility. Mutations in this gene are associated with epidermolysis bullosa with pyloric atresia, and its heterodimer, α6β4, plays a role in the binding of IGF1, which is itself essential for IGF1 signaling. Based on the identification of these three variants in all the available affected family members, one possible hypothesis is that they act following an oligogenic model of inheritance. Nevertheless, based on data available in literature as well as the data accumulated in the current thesis, it is difficult to link the genes mutated in this family to the predicted mechanisms of pathogenesis for OAVS. Further work, based on functional studies in animal and cellular models as well as investigation of larger sample cohorts may help to find an explanation for current findings in this family. It has also to be considered that some apparently unaffected members of the family, did carry the three variants together, a finding implying that other genes or mechanisms could be related to the OAVS family phenotype.

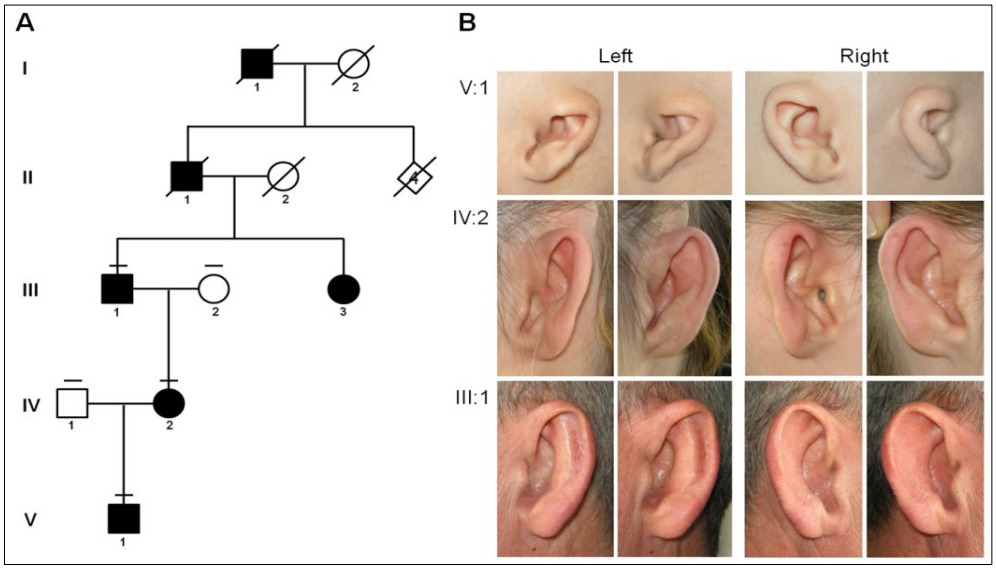
The OAVS spectrum is broad, ranging from isolated microtia to Goldenhar's syndrome. Microtia and OAVS share the following characteristics: 1) variable phenotypic expression, 2) asymmetric involvement of facial structures, 3) right side preponderance, 4) male predilection, and 5) familial occurrence of microtia or related anomalies such as preauricular tags and pits. Based on these observations, it has been suggested that isolated microtia represents a milder phenotype of OAVS (Llano-Rivas et al., 1999 ; Rollnick and Kaye., 1983 ; Tasse et al., 2005). In a Turkish population with GS, microtia was found in 52% of patients. This has led to the controversial concept that most (or all) cases presenting with apparent isolated microtia are actually cases of OAVS. This controversy remains unsettled. In many cases, the occurrence of microtia associated with chromosomal abnormalities and in single gene disorders supports a complex genetic regulatory network coordinating morphogenesis of the external ear. Therefore, although the clinical expression

of microtia and OAVS overlap and likely share many common genetic mechanisms, each should be considered as a separate entity. From this perspective, It has also been studied a five-generation family with isolated bilateral microtia segregating as an autosomal dominant trait.These clinical observations confirm the concept that microtia and hemifacial microsomia have the same etiopathogenesis which is not shared by mandibulofacial dysostosis (Bennun et al., 1985).

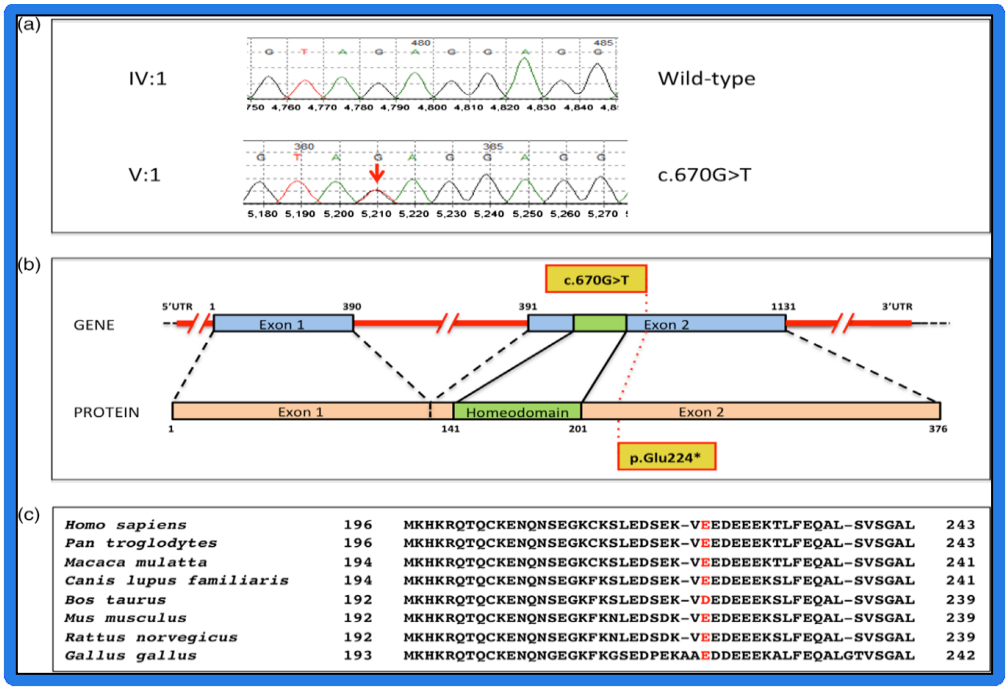
The index patient (V:1; Figure 16) was the first child of Italian non-consanguineous parents. Gestational and family history were unremarkable. His ears were overtly dysplastic. In particular, they showed decreased median longitudinal length in presence of mildly sharp pointed superior portion of the helix, absent superior crus and triangular fossa of the antihelix, overfolded helix with an underdeveloped inferior crus, underdeveloped antitragus, serpiginous antihelix stem, deep incisura between tragus and antitragus with a laterally dislocated hypoplastic lobe. Other outer ear structures and canals were average. Otoacoustic emissions were bilaterally regular.

Index patient's mother (IV:2; Figure 16) had similar ear anomaly, with the except of a normally folded helix, markedly sharp pointed superior portion and underdeveloped antihelix stem with Satyr appearance. The ears of the maternal grandfather (III:1;Figure 16) had a normal median longitudinal length but showed a distinctive appearance with markedly flat antihelix, less represented inferior crus of the helix, broad incisura between tragus and antitragus, lateralization of the lobe and poor differentiation among lobe, antitragus and descending part of the helix. In both relatives audiometric testing had unremarkable results, as well as intraoral examination.

Extended family history disclosed dysplastic ears also in a maternal second-degree aunt (III:3; Figure 16), the maternal great-grandfather (II:1;Figure 16) and great-great-grandfather (I:1; Figure 16). Owing to the absence of any hearing difficulty at audiometry, examined family members refused further investigations.



Sanger analysis of the index patient (V:1) identified a previously unreported heterozygous variant [NM_006735.3: c.670G>*T,* p.(Glu224*)] in the second exon of the *HOXA2* gene (Figure 17a). This variant was predicted to introduce a premature stop codon at amino acid position 224 and result in truncation of 153 amino acids from the C-terminal end of the HOXA2 protein (Figure 17b) and may pathogenetically act either via haploinsuffieciency (i.e. PTC NMD permitted) or gain/loss of function, or a mixture of both. Sanger sequencing confirmed no additional non-synonymous variant in either *HOXA2 alleles.* Moreover, mutation analysis, extended to other family members (III:1, III:2, IV:1 and IV:2), confirmed co-segregation of the variant with disease. This variant was not annotated in public databases for human genetic variants. Furthermore, analysis of evolutionary conservation on the protein residue level revealed that the mutated amino acid is within a region of high-evolutionary conservation (Figure 18c).



This is the second autosomal dominant family with non-syndromic microtia with mutation in *HOXA2* suggesting that the hallmarks of *HOXA2* perturbations may be considered the presence of isolated microtia featuring a non-alignment between a broad incisura and lobe attachment with or without mixed deafness, but lacking inner ear anomalies and microdontia. Present and previous findings provide compelling evidence that *HOXA2* mutations might cause a potentially discrete form of microtia (Alasti et al., 2008 ; Brown et al., 2013 ; Piceci et al., 2016).Compared with the recessive family with homozygous missense *HOXA2* mutation (Alasti et al., 2008), perturbation of middle ear development seems less constant in dominant families, as deafness was reported only in some family members by Brown et al. and is nearly absent in our patients (Brown et al., 2013). Considering the high phenotypical heterogeneity of OAVS, showing in some cases, exclusively microtia and taken into account that *Hoxa2* is involved in the genetic pathway of branchial arch development, suggest that *Hoxa 2* gene belongs to the molecular pattern of candidate genes underlying OAVS.

6. Conclusions

In conclusion, the current study carried out on a cohort including a total of 84 OAVS individuals has contributed to:

1) Detect new genomic rearrangements enlarging the spectrum of genetic abnormalities associated to OAVS as well as confirming the importance of the SNP-Array approach in genetic definition of these patients.

2) Identify a novel missense variant in *Gosecoid* gene, which was previously reported as a candidate gene for OAVS by linkage analysis, as well as previously unreported mutations in a number of new genes related to disorders with phenotypic overlapping with OAVS.

3) Identify candidate variants in new genes belonging to molecular pathways contributing to development of derivatives of the first and second branchial arches.

4) Confirm the presence of three new mutations in the *MYT1* gene, which was recently proposed as a the first causative gene for OAVS, based on its participation to the RA pathway.

5) Described the second nonsense mutation in the *HOXA2* gene in a family with autosomal dominant non-syndromic microtia and distinctive ear morphology.

On the whole, the data collected in this thesis represent a unique work providing new insights into the genetic mechanisms underlying OAVS, a condition that remains largely unexplained in its etiology.

7. Bibliografy

Acampora D, Merlo GR, Paleari L, Zerega B, Postiglione MP, Mantero S, Bober E, Barbieri O, Simeone A, Levi G. (1999) "Craniofacial, vestibular and bone defects in mice lacking the *Distal-less*-related gene *Dlx5". Development*. 126, pp. 3795–3809.

Ahlgren SC, Bronner-Fraser M. (1999). "Inhibition of Sonic hedgehog signaling in vivo results in craniofacial neural crest cell death". *Curr. Biol.* 9: 1304–1314.

Akam M. (1989) "Hox and HOM: homologous gene clusters in insects and vertebrates". *Cell*. 57(3):347-9.

Akazawa H, Komuro I, Sugitani Y, Yazaki Y, Nagai R, Noda T. (2000)"Targeted disruption of the homeobox transcription factor Bapx1 results in lethal skeletal dysplasia with asplenia and gastroduodenal malformation". *Genes Cells.* 5:499–513.

Ala-Mello S1, Siggberg L, Knuutila S, von Koskull H, Taskinen M, Peippo M.(2008) "Further evidence for a relationship between the 5p15 chromosome region and the oculoauriculovertebral anomaly". *Am J Med Genet A*.1;146A(19):2490-4.

Alasti F, Sadeghi A, Sanati MH, Farhadi M, Stollar E, Somers T, Van Camp G.(2008) "A mutation in HOXA2 is responsible for autosomal-recessive microtia in an Iranian family". *Am J Hum Genet.* 83: 424.

Aleksic S, Budzilovich G, Greco MA, McCarthy J, Reuben R, Margolis S, Epstein F, Feigin I, Pearson J. (1984) "Intracranial lipomas, hydrocephalus and other CNS anomalies in oculoauriculovertebral dysplasia (Goldenhar-Gorlin syndrome)". *Childs Brain*. 11:285–297.

Alfi D, Lam D, Gateno J. (2014) "Branchial arch syndromes". *Atlas Oral Maxillofac Surg Clin North Am*. 22(2):167-73.

Anderson PJ, David DJ. (2005) "Spinal anomalies in Goldenhar syndrome". *Cleft Palate Craniofac J*. 42(5):477-80.

Arzimanoglou AA, Salefranque F, Goutières F, Aicardi J. (1999) "Hemifacial spasm or subcortical epilepsy?". *Epileptic Disord*. 1:121–125.

Ballesta-Martinez MJ, Lopez-Gonzalez V, Dulcet LA, Rodriguez-Santiago B, Garcia-Minaur S, Guillen-Navarro E.(2013) "Autosomal dominant oculoauriculovertebral spectrum and 14q23.1 microduplication". *Am J Med Genet A*. 161A:2030–2035.

Barisic I, Odak L, Loane M, Garne E, Wellesley D, Calzolari E, Dolk H, Addor MC, Arriola L, Bergman J, Bianca S, Doray B, Khoshnood B,Klungsoyr K, McDonnell B, Pierini A, Rankin J, Rissmann A, Rounding C, Queisser-Luft A, Scarano G, Tucker D. (2014) "Prevalence, prenatal diagnosis and clinical features of oculo-auriculo-vertebral spectrum: a registry-based study in Europe". *Eur J Hum Genet*. 1-8.

Beleza-Meireles A, Hart R, Clayton-Smith J, Oliveira R, Reis CF, Venâncio M, Ramos F, Sá J, Ramos L, Cunha E, Pires LM, Carreira IM, Scholey R, Wright R, Urquhart JE, Briggs TA, Kerr B, Kingston H, Metcalfe K, Donnai D, Newman WG, Saraiva JM, Tassabehji M. (2015) "Oculo-auriculo-vertebral spectrum: clinical and molecular analysis of 51 patients". *Eur J Med Genet*. 58:455–465.

Beltinger C, Saule H. (1988) "Imaging of lipoma of the corpus callosum and intracranial dermoids in the Goldenhar syndrome". *Pediatr Radiol*. 18:72–73.

Bennun RD, Mulliken JB, Kaban LB, Murray JE.(1985) "Microtia: a microform of hemifacial microsomia". *Plast Reconstr Surg*. 76(6):859-65.

Brotto D , Manara R , Ghiselli S, Lovo E, Mardari R, Toldo I, Castiglione A, Schifano G, Stritoni V, Bovo R, Trevisi P, Martini A. (2017) "Oculo-Auriculo-Vertebral Spectrum: Going Beyond the First and Second Pharyngeal Arch Involvement" *Neuroradiology*. 59 (3), 305-316.

Brown KK, Viana LM, Helwig CC, Artunduaga MA, Quintanilla-Dieck L, Jarrin P, Osorno G, McDonough B, DePalma SR, Eavey RD, Seidman JG, Seidman CE.(2013) "HOXA2 haploinsufficiency in dominant bilateral microtia and hearing loss". *Hum Mutat*. 34:1347–1351.

Bulfone A, Puelles L, Porteus MH, Frohman MA, Martin GR, Rubenstein JLR. (1993) "Spatially restricted expression of Dlx-1, Dlx-2 (Tes-1), Gbx-2, and Wnt-3 in the embryonic day 12.5 mouse forebrain defines potential transverse and longitudinal segmental boundaries". *J. Neurosci*. 13, 3155-3172.

Chai L, Yang J, Di C, Cui W, Lai R, Ma Y. (2006) "Transcriptional activation of the sall1 by the human six1 homeodomain during kidney development". *The Journ of Biol Chem*. 281, 18918-18926.

Chai Y, Maxson RE Jr. (2006) "Recent advances in craniofacial morphogenesis". *Dev Dyn*. 235(9):2353-75.

Chiang C, Litingtung Y, Lee E, Young KE, Corden JL, Westphal H, Beachy PA. (1996) "Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function". *Nature*. 383(6599):407-13.

Chong PF, Haraguchi K, Torio M, Kirino M, Ogata R, Matsukura M, Sakai Y, Ishizaki Y, Yamamoto T, Kira R. (2015) "A case of pontine tegmental cap dysplasia with comorbidity of oculoauriculovertebral spectrum". *Brain and Development*. 37:171–174.

Clementi M, Tenconi R, Turolla L, Silvan C, Bortotto L, Artifoni L.(1991) "Apparent CHARGE association and chromosome anomaly: chance or contiguous gene syndrome". *Am J Med Genet*. 41(2):246–50.

Cohn MJ.(2002) "Evolutionary biology: lamprey Hox genes and the origin of jaws". *Nature*. 28;416(6879):386-7.

Conrad DF, Bird C,Blackburne B, Lindsay S, Mamanova L, Lee C, Turner DJ, Hurles ME. (2010) "Mutation spectrum revealed by breakpoint sequencing of human germline CNVs". *Nat Genet*. 42(5): 385–391.

Couly G, Creuzet S, Bennaceur S, Vincent C, Le Douarin NM. (2002) "Interactions between Hox-negative cephalic neural crest cells and the foregut endoderm in patterning the facial skeleton in the vertebrate head". *Development*. 129:1061–1073.

Cousley R.R. (1993) "A comparison of two classification systems for hemifacial microsomia". *Br. J. Oral. Maxillofac. Surg.* 31 (2), pp. 78–82.

Cox TC, Camcia ED, Voraa S, Luquettia DV, Turnerb EE. (2014) "The genetics of auricular development and malformation: New findings in model systems driving future directions for microtia research" *Eur J Med Genet*. 57:394-401.

Dauber A, Golzio C, Guenot C, Jodelka FM, Kibaek M, Kjaergaard S, Leheup B, Martinet D, Nowaczyk MJ, Rosenfeld JA, Zeesman S, Zunich J, Beckmann JS, Hirschhorn JN, Hastings ML, Jacquemont S, Katsanis N.(2013) "SCRIB and PUF60 are primary drivers of the multisystemic phenotypes of the 8q24.3 copy-number variant". *Am J Hum Genet.*7;93(5):798-811.

Dauwerse JG, Dixon J, Seland S, Ruivenkamp CA, van Haeringen A, Hoefsloot LH, Peters DJ, Boers AC, Daumer-Haas C, Maiwald R, Zweier C, Kerr B, Cobo AM, Toral JF, Hoogeboom AJ, Lohmann DR, Hehr U, Dixon MJ, Breuning MH, Wieczorek D. (2011) "Mutations in genes encoding subunits of RNA polymerases I and III cause Treacher Collins syndrome". *Nat Genet* . 43:20–22.

De Krijger RR, Mooy CM, Van Hemel JO, Sulkers EJ, Kros JM, Bartelings MM, Govaerts LC.(1999) "CHARGE association-related ocular pathology in a newborn with partial trisomy 19q and partial monosomy 21q, from a maternal translocation (19;21)(q13.1;q22.3)". *Pediatr Dev Pathol*. 2(6):577–81.

De Ravel TJ, Legius E, Brems H, Van Hoestenberghe R, Gillis PH, Fryns JP. (2001). "Hemifacial microsomia in two patients further supporting chromosomal mosaicism as a causative factor". *Clin Dysmorphol.* 10, 263–267.

Depew MJ, Lufkin T, Rubenstein JL. (2002) "Specification of jaw subdivisions by *Dlx* genes". *Science*. 298, pp. 381–385.

Depew MJ, Liu JK, Long JE, Presley R, Meneses JJ, Pedersen RA, Rubenstein JL. (1999) "*Dlx5* regulates regional development of the branchial arches and sensory capsules". *Development*. 126, pp. 3831–3846.

Di Leone RJ, Russell LB, Kingsley DM. (1998) "An extensive 30 regulatory region controls expression of Bmp5 in specific anatomical structures of the mouse embryo." *Genetics*.148:401-8.

Digilio MC, McDonald-McGinn DM, Heike C, Catania C, Dallapiccola B, Marino B, Zackai EH. (2009) "Three patients with oculo-auriculo-vertebral spectrum and microdeletion 22q11.2". *Am J Med Genet A*. 149A(12):2860-4.

Dixon J, Ellis I, Bottani A, Temple K, Dixon MJ. (2004) "Identification of mutations in TCOF1: Use of molecular analysis in the pre- and postnatal diagnosis of Treacher Collins syndrome". *Am J Med Genet A*. 127A: 244–248.

Ewart-Toland A, Yankowitz J, Winder A, Imagire R, Cox VA, Aylsworth AS. (2000) "Oculoauriculovertebral abnormalities in children of diabetic mothers." *Am J Med Genet*. 14;90(4):303-9.

Fabrizio P, Laggerbauer B, Lauber J, Lane WS, Lührmann R. (1997) "An evolutionarily conserved U5 snRNP-specific protein is a GTP-binding factor closely related to the ribosomal translocase EF-2". *EMBO J*. 16 (13): 4092-4106.

Farra C, Yunis K, Yazbeck N, Majdalani M, Charafeddine L, Wakim R, Awwad J. (2011) "A Lebanese family with autosomal recessive oculo-auriculo-vertebral (OAV) spectrum and review of the literature: is OAV a genetically heterogeneous disorder?" *Appl Clin Genet*. 6;4:93-7.

Fischer S, Lüdecke HJ, Wieczorek D, Böhringer S, Gillessen-Kaesbach G, Horsthemke B. (2006) "Histone acetylation dependent allelic expression imbalance of BAPX1 in patients with the oculo-auriculo-vertebral spectrum". *Hum Mol Genet*.15(4):581-7.

Franco PG, Paganelli AR, Lopez SL, Carrasco AE (1999). "Functional association of retinoic acid and hedgehog signaling in Xenopus primary neurogenesis". *Development*.126:4257–4265.

Fujimura M, Sonobe S, Nishijima Y, Niizuma K, Sakata H, Kure S, Tominaga T. (2014) "Genetics and Biomarkers of Moyamoya Disease: Significance of RNF213 as a Susceptibility Gene". *J Stroke*. 16(2):65-72.

Gamsjaeger R, O'Connell MR, Cubeddu L, Shepherd NE, Lowry JA, Kwan AH, Vandevenne M, Swanton MK, Matthews JM, Mackay JP. (2013) "A structural analysis of DNA binding by myelin transcription factor 1 double zinc fingers". *J Biol Chem*. 288:35180–91.

Gaunt SJ, Blum M, De Robertis EM. (1993) "Expression of the mouse goosecoid gene during mid-embryogenesis may mark mesenchymal cell lineages in the developing head, limbs and body wall". *Development*. 117, pp. 769–778.

Gelboin HV, Krausz K . (2006). "Monoclonal antibodies and multifunctional cytochrome P450: drug metabolism as paradigm". *J. Clin. Pharmacol.*46:353 – 372 .

Gerbal-Chaloin S,. , Pascussi JM, Pichard-Garcia L , Daujat M , Waechter F, Fabre JM , Carrere N, Maurel P.(2001) . "Induction of CYP2C genes in human hepatocytes in primary culture". *Drug Metab. Dispos.* 29: 242 – 251.

Gerkes EH, van Ravenswaaij CM, van Essen AJ.(2008) "Question mark ears and post-auricular tags". *Eur J Med Genet.* 51:264–7.

Goldenhar M. (1952) "Associations malformatives do I'oeil et de I'oreille, en particulier le syndrome dermo¨ide epidbulbaire – appendices auricularies – fistula auris congenita et ses relations avec la dysostose mandibulo-faciale". *J Genet Hum*. 1: 243–82.

Goodin K, Prucka S, Woolley AL, Kohlhase J, Smith RJ, Grant J, Robin NH. (2009) "Familial transmission of oculoauriculovertebral spectrum (Goldenhar syndrome) is not due to mutations in either EYA1 or SALL1". *Am J Med Genet*.149A(3):535-8.

Gordon CT, Petit F, Oufadem M, Decaestecker C, Jourdain AS, Andrieux J, Malan V, Alessandri JL, Baujat G, Baumann C, Boute-Benejean O, Caumes R, Delobel B, Dieterich K, Gaillard D, Gonzales M, Lacombe D, Escande F, Manouvrier-Hanu S, Marlin S, Mathieu-Dramard M, Mehta SG, Simonic I, Munnich A, Vekemans M, Porchet N, de Pontual L, Sarnacki S, Attie-Bitach T, Lyonnet S, Holder-Espinasse M, Amiel J. (2012) "EFTUD2 haploinsufficiency leads to syndromic oesophageal atresia". *J Med Genet*. 49:737–46.

Gorlin RJ, Cohen MM, Levin LS. (1990) "Syndromes of the Head and Neck." New York: Oxford University Press, pp 641±649, 707±708.

Gorlin RJ, Hennekam R, Allanson J, Krantz I. (2010) "Gorlin's syndromes of the Head and Neck 5th edition". New York: Oxford University Press. pp. 790–8.

Gorlin RJ, Meskin LH, Peterson WC, Goltz RW. (1963) "Focal dermal hypoplasia syndrome". *Acta Derm Venereol*. 43:421–40.

Gorlin RJ,Pindborg J, Cohen MM Jr. (1976) "Oculoauricolarvertebral dysplasia.In:Syndromes of the head and neck. 2nd ed". New-York:McGraw-Hill : 546-552.

Gorlin RJ. (2001). "Syndromes of the Head and Neck. 3rd edition". In: Gorlin RJCMJ, Hennekam RCM, editors. Oxford: Oxford University Press. p 790–849.

Graham A. (2003) "Development of the pharyngeal arches". *Am J Med Genet* A. 119:251–256.

Graham JM Jr. (2001) "A recognizable syndrome within CHARGE association: HallHittner syndrome". *Am J Med Genet*. 99(2):120–3.

Grammatopoulos GA, Bell E, Toole L, Lumsden A, Tucker AS.(2000) "Homeotic transformation of branchial arch identity after Hoxa2 overexpression". *Development*. 127(24):5355-65.

Grigoriou M, Tucker AS, Sharpe PT, Pachnis V. (1998) "Expression and regulation of Lhx6 and Lhx7, a novel subfamily of LIM homeodomain encoding genes, suggests a role in mammalian head development". *Development*. 125:2063–2074.

Grix A Jr. Am J. (1982) "Malformations in infants of diabetic mothers".*Med Genet*.13(2):131-7.

Gu G, Wells JM, Dombkowski D, Preffer F, Aronow B, Melton DA.(2004) "Global expression analysis of gene regulatory pathways during endocrine pancreatic development". *Development*. 131(1):165-79.

Guida V, Sinibaldi L, Pagnoni M, Bernardini L, Loddo S, Margiotti K, Digilio MC, Fadda MT, Dallapiccola B, Iannetti G, De Luca A (2014) "A *de novo* proximal 3q29 chromosome microduplication in a patient with oculo auriculo vertebral spectrum" *Am J Med Genet A*. 167(4):797-801.

Gustavson EE, Chen H. (1985) "Goldenhar syndrome, anterior encephalocele, and aqueductal stenosis following fetal primidone exposure". *Teratology*. 32:13–17.

Haberland M, Mokalled MH, Montgomery RL, Olson EN. (2009) "Epigenetic control of skull morphogenesis by histone deacetylase 8." *Genes Dev*. 23(14):1625-30.

Hartsfield JK. (2007) "Review of the etiologic heterogeneity of the oculo-auriculo-vertebral spectrum (Hemifacial Microsomia)". *Orthod Craniofacial Res*. 10(3):121-8.

Heanue TA, Davis RJ, Rowitch DH, Kispert A, McMahon AP, Mardon G, Tabin CJ. (2002). "Dach1, a vertebrate homologue of Drosophila dachshund, is expressed in the developing eye and ear of both chick and mouse and is regulated independently of Pax and Eya genes". *Mech Dev.* 111:75–87.

Hehir-Kwa JY1, Pfundt R, Veltman JA, de Leeuw N. (2013) "Pathogenic or not? Assessing the clinical relevance of copy number variants" *Clin Genet*.84(5):415-21.

Heike CL, Luquetti DV, Hing AV. "Craniofacial Microsomia Overview".In: Pagon RA, Adam MP, Ardinger HH, Wallace SE, Amemiya A, Bean LJH, Bird TD, Ledbetter N, Mefford HC, Smith RJH, Stephens K, editors. GeneReviews® [Internet]. Seattle (WA): University of Washington, Seattle; 1993-2017. 2009 Mar 19 [updated 2014 Oct 9].

Hennekam RCM, Krantz ID, Allanson JE. (2010) "Branchial arch and oralacral disorders. In: Gorlin's syndromes of the head and neck, 5th edition". New York: Oxford University Press Inc. pp 879–887.

Herman GE, Greenberg F, Ledbetter DH. (1988) "Multiple congenital anomaly/mental retardation (MCA/MR) syndrome with Goldenhar complex due to a terminal del(22q)." *Am J Med Genet*.(4):909-15.

Hillig T, Krustrup P, Fleming I, Osada T, Saltin B, Hellsten Y.(2003) "Cytochrome P450 2C9 plays an important role in the regulation of exercise-induced skeletal muscle blood flow and oxygen uptake in humans". *J Physiol*. 546(Pt 1):307–314.

Huang XS; Xiao L, Li X, Xie Y, Jiang H, Tan C,Wang L, Zhang JX.(2010) “Two neighboring microdeletions of 5q13.2 in a child with oculo-auriculo-vertebral spectrum”. *Eur J Med Genet*. 53(3):153-8.

Hunt P, Krumlauf R.(1991) “Hox genes coming to a head”.*Curr Biol*.1(5):304-6.

Hunter A, Frias JL, Gillessen-Kaesbach G, Hughes H, Jones KL, Wilson L.(2009) “Elements of morphology: standard terminology for the ear”. *Am J Med Genet A*. 149A: 40–60.

Hurst JA, Meinecke P, Baraitser M.(1991) “Balanced t(6;8)(6p8p;6q8q) and the CHARGE association”. *J Med Genet*. 28(1):54–5.

Jeanty P, Zaleski W, Fleischer AC. (1991) “Prenatal sonographic diagnosis of lipoma of the corpus callosum in a fetus with Goldenhar syndrome”. *Am J Perinatol*. 8:89–90.

Jena AK, Duggal R. (2006) “Atypical Goldenhar syndrome: a case report”. *J Clin Pediatr Dent*. 31:118–122.

Jeong J, Mao J, Tenzen T, Kottmann AH, Mcmahon AP. (2004) “Hedgehog signaling in the neural crest cells regulates the patterning and growth of facial primordial”. *Genes Dev*. 18, pp. 937–951.

Josifova DJ, Patton MA, Marks K. (2004) “Oculoauriculovertebral spectrum phenotype caused by an unbalanced t(5;8)(p15.31;p23.1) rearrangement”. Clin *Dysmorphol*. 13(3):151-3.

Katsanis SH, Jabs EW. (2012) “Treacher Collins Syndrome”. *GeneReviews™*. Seattle, WA: University of Washington.

Kaye CI, Martin AO, Rollnick BR, Nagatoshi K, Israel J, Hermanoff M, Tropea B, Richtsmeier JT, Morton NE. (1992) “Oculoauriculovertebral anomaly: segregation analysis”. *Am J Med Genet*. 43:913–917

Kelberman D, Tyson J, Chandler DC, McInerney AM, Slee J, Albert D, Aymat A, Botma M, Calvert M, Goldblatt J, Haan EA, Laing NG, Lim J, Malcolm S, Singer SL, Winter RM, Bitner-Glindzicz M. (2001) “Hemifacial microsomia: progress in understanding the genetic basis of a complex malformation syndrome”. *Hum Genet*. 109(6):638-45.

Kerckoff Villanueva H, Retamoza B, Bautista A. (2008) “Diabetic mother's newborn with Goldenhar syndrome and cerebral malformations. Case report”. *Ginecol Obstet Mex*. 76:691–694

Khaja R, Zhang J, MacDonald JR, He Y, Joseph-George AM, Wei J, Rafiq MA, Qian C, Shago M, Pantano L, Aburatani H, Jones K, Redon R, Hurles M, Armengol L, Estivill X, Mural RJ, Lee C, Scherer SW, Feuk L.(2006) "Genome assembly comparison identifies structural variants in the human genome." *Nat Genet*. 38(12):1413-8.

Kim JG, Armstrong RC, v Agoston D, Robinsky A, Wiese C, Nagle J, Hudson LD.(1997) "Myelin transcription factor 1 (Myt1) of the oligodendrocyte lineage, along with a closely related CCHC zinc finger, is expressed in developing neurons in the mammalian central nervous system". *J Neurosci Res*.50:272–90.

Klockars T, Suutarla S, Kentala E, Ala-Mello S, Rautio J.(2007). " Inheritance of microtia in the Finnish population". *Int J Pediatr Otorhinolaryngol*. 71(11):1783-8.

Kohlhase J. (2007) "Townes-Brocks Syndrome". *GeneReviews™*. Seattle, WA: University of Washington.

Kroepfl T, Petek E, Schwarzbraun T, Kroisel PM, Plecko B. (2008) "Mental retardation in a girl with a subtelomeric deletion on chromosome 20q and complete deletion of the myelin transcription factor 1 gene (MYT1)". *Clin Genet*.73:492–5.

Kumar R, Balani B, Patwari AK, Anand VK, Ahuja B. (2000) "Goldenhar syndrome with rare associations". *Indian J Pediatr*. 67: 231–233

Lafay-Cousin L, Payne E, Strother D, Chernos J, Chan M, Bernier FP. (2009) "Goldenhar phenotype in a child with distal 22q11.2 deletion and intracranial atypical teratoid rhabdoid tumor". *Am J Med Genet A*. 149:2855–2859

Lalani SR, Hefner MA, Belmont JW, Davenport SLH. (2006) "CHARGE Syndrome". *GeneReviews™*. Seattle, WA: University of Washington.

Lalani SR, Safiullah A, Molinari L, Fernbach S, Martin D, Belmont J. (2004) "SEMA3E mutation in a patient with CHARGE syndrome" *J Med Genet*. 41(7): e94.

Lalani SR, Stockton DW, Bacino C, Molinari LM, Glass NL, Fernbach SD, Towbin JA, Craigen WJ, Graham JM Jr, Hefner MA, Lin AE, McBride KL, Davenport SL, Belmont JW.(2003) "Toward a genetic etiology of CHARGE syndrome: I. A systematic scan for submicroscopic deletions". *Am J Med Genet*.118A(3):260–6.

Lammer EJ, Chen DT, Hoar RM, Agnish ND, Benke PJ, Braun JT. (1985) "Retinoic acid embryopathy". *N Engl J Med*. 313(14):837-41

Lee TV1, Sethi MK, Leonardi J, Rana NA, Buettner FF, Haltiwanger RS, Bakker H, Jafar-Nejad H. (2013) "Negative regulation of notch signaling by xylose." *PLoS Genet*. 9(6):e1003547.

Lettice L, Hecksher-Sorensen J, Hill R. (2001) "The role of Bapx1 (Nkx3.2) in the development and evolution of the axial skeleton*". J Anat*.199:181–187.

Lev D, Nakar O, Bar-Am I, Zudik A, Watemberg N, Finkelstien S, Katzin N, Lerman-Sagie T.(2000) "CHARGE association in a child with de novo chromosomal aberration 46, X,der(X)t(X;2)(p22.1;q33) detected by spectral karyotyping". *J Med Genet*. 37(12):E47.

Limdi NA, Arnett DK, Goldstein JA, Beasley TM, McGwin G, Adler BK, Acton RT. (2008)b"Influence of CYP2C9 and VKORC1 on warfarin dose, anticoagulation attainment and maintenance among European-Americans and African-Americans". *Pharmacogenomics*. 9(5):511–526.

Limdi NA, McGwin G, Goldstein JA, Beasley TM, Arnett DK, Adler BK, Baird MF, Acton RT(2008)a. "Influence of CYP2C9 and VKORC1 1173C/T genotype on the risk of hemorrhagic complications in African-American and European-American patients on warfarin". *Clin Pharmacol Ther*. 83(2):312–321.

Lin JHC, Kobari Y, Zhu Y, Stemerman MB, Pritchard KAJ.(1996) "Human umbilical vein endothelial cells express P450 2C8 mRNA:cloning of endothelial P450 epoxygenase". *Endothelium*. 4:219–229.

Lindsley D, Sandler L, Baker BS, Carpenter AT, Denell RE, Hall JC, Jacobs PA, Miklos GL, Davis BK, Gethmann RC, Hardy RW, Steven AH, Miller M, Nozawa H, Parry DM, Gould-Somero M, Gould-Somero M. (1972) "Segmental aneuploidy and the genetic gross structure of the Drosophila genome." *Genetics*.71(1):157-84.

Lines MA, Huang L, Schwartzentruber J, Douglas SL, Lynch DC, Beaulieu C, Guion-Almeida ML, Zechi-Ceide RM, Gener B, Gillessen-Kaesbach G, Nava C, Baujat G, Horn D, Kini U, Caliebe A, Alanay Y, Utine GE, Lev D, Kohlhase J, Grix AW, Lohmann DR, Hehr U, Böhm D; FORGE Canada Consortium, Majewski J, Bulman DE, Wieczorek D, Boycott KM.(2012) "Haploinsufficiency of a spliceosomal GTPase encoded by EFTUD2 causes mandibulofacial dysostosis with microcephaly". *Am J Hum Genet*. 90 (2): 369-377.

Liu W, Morito D, Takashima S, Mineharu Y, Kobayashi H, Hitomi T, Hashikata H, Matsuura N, Yamazaki S, Toyoda A, Kikuta K, Takagi Y, Harada KH, Fujiyama A, Herzig R, Krischek B, Zou L, Kim JE, Kitakaze M, Miyamoto S, Nagata K, Hashimoto N, Koizumi A.(2011) "Identification of RNF213 as a susceptibility gene for moyamoya disease and its possible role in vascular development". *PLoS One*.6(7):e22542.

Llano-Rivas I, Gonza´lez-del Angel A, del Castillo V, Reyes R, Carnevale A.(1999) "Microtia: a clinical and genetic study at the National Institute of Pediatrics in Mexico City". *Arch Med Res*. 30:120–124.

Lopez E, Berenguer M, Tingaud-Sequeira A, Marlin S, Toutain A, Denoyelle F, Picard A, Charron S, Mathieu G, de Belvalet H, Arveiler B, Babin PJ, Lacombe D, Rooryck C. (2016) "Mutations in MYT1, encoding the myelin transcription factor 1, are a rare cause of OAVS". *J Med Genet*. pii: jmedgenet-2016-103774.

Lupski JR. (2007) "Genomic rearrangements and sporadic disease" *Nat Genet*. 39 (7 Suppl):S43-7.

Masotti C, Oliveira KG, Poerner F, Splendore A, Souza J, Rda S Freitas, Zechi-Ceide R, Guion-Almeida ML, Passos-Bueno MR. (2008) "Auriculo-condylar syndrome: mapping of a first locus and evidence for genetic heterogeneity". *Eur J Hum Genet*. 16:145–52.

Massague J, Chen YG. (2000) "Controlling TGF-beta signaling." *Genes Dev*. 14, pp. 627–644

Matsuo, Kuratani S, Rimura C, Takeda and Aizawa. (1995) "Mouse Otx2 functions in the formation and patterning of rosrtal head". *Genes Dev*. 9: 2646-2658.

McCarroll SA1, Hadnott TN, Perry GH, Sabeti PC, Zody MC, Barrett JC, Dallaire S, Gabriel SB, Lee C, Daly MJ, Altshuler DM. (2006) "Common deletion polymorphisms in the human genome." International HapMap Consortium. *Nat Genet.* (1):86-92.

McGowan R, Murday V, Kinning E, Garcia S, Koppel D, Whiteford M. (2011) "Novel features in auriculo-condylar syndrome". *Clin Dysmorphol*. 20:1–10.

Miller KA, Tan TY, Welfare MF, White SM, Stark Z, Savarirayan R, Burgess T,Heggie AA, Caruana G, Bertram JF, Bateman JF, Farlie PG. (2014) "A mouse splice-site mutant and individuals with atypical chromosome 22q11.2 deletions demonstrate the crucial role for crkl in craniofacial and pharyngeal development". *Mol. Syndromol*. 5 (6), 276e286.

Minoux M, Rijli FM. (2010)"Molecular mechanisms of cranial neural crest cell migration and patterning in craniofacial development". *Development* (Cambridge, England). 137(16):2605–2621.

Minoux M, Kratochwil CF, Ducret S, Amin S, Kitazawa T, Kurihara H, Bobola N, Vilain N, Rijli FM. (2013) "Mouse Hoxa2 mutations provide a model for microtia and auricle duplication." *Development*.140:4386-97.

Monks DC, Morrow BE.(2012) "Identification of putative retinoic acid target genes downstream of mesenchymal Tbx1 during inner ear development". *Dev Dyn*. 241(3):563-73.

Moore-Scott BA, Manley NR. (2005) "Differential expression of Sonic hedgehog along the anterior–posterior axis regulates patterning of pharyngeal pouch endoderm and pharyngeal endoderm-derived organs". *Dev. Biol*. 278, pp. 323–33.

Naora H, Kimura M, Otani H, Yokoyama M, Koizumi T, Katsuki M, Tanaka O. (1994) "Transgenic mouse model of hemifacial microsomia: cloning and characterization of insertional mutation region on chromosome 10." *Genomics*. (3):515-9.

Need AC, Shashi V, Hitomi Y, Schoch K, Shianna KV, McDonald MT, Meisler MH, Goldstein DB.(2012) "Clinical application of exome sequencing in undiagnosed genetic conditions". *J Med Genet*. 49 (6): 353-361.

North KN, Wu BL, Cao BN, Whiteman DA, Korf BR.(1995) "CHARGE association in a child with de novo inverted duplication (14)(q22Rq24.3)". *Am J Med Genet*. 57(4):610–4.

Ou Z, Martin DM, Bedoyan JK, Cooper ML, Chinault AC, Stankiewicz P, Cheung S.W. (2008) "Branchiootorenal Syndrome and Oculoauriculovertebral Spectrum Features Associated With Duplication of SIX1, SIX6, and OTX2 Resulting From a Complex Chromosomal Rearrangement". *Am J Med Genet*. 146A:2480–2489.

Pan W, Jin Y, Chen J, Rottier RJ, Steel KP, Kiernan AE. (2013). "Ectopic expression of activated notch or SOX2 reveals similar and unique roles in the development of the sensory cell progenitors in the mammalian inner ear". *J Neurosci*. 33:16146–16157.

Pane M, Baranello G, Battaglia D, Donvito V, Carnevale F, Stefanini MC, Guzzetta F, Mercuri E, Bertini E. (2004) “Severe abnormalities of the pons in two infants with Goldenhar syndrome”. *Neuropediatrics*. 35:234–238.

Petryk A, Anderson RM, Jarcho MP, Leaf I, Carlson CS, Klingensmith J, Shawlot W, O’Connor MB. (2004) “The mammalian twisted gastrulation gene functions in foregut and craniofacial development.” *Dev. Biol*. 267:374e86.

Piceci F, Morlino S, Castori M, Buffone E, De Luca A, Grammatico P, Guida V. (2017)“Identification of a second HOXA2 nonsense mutation in a family with autosomal dominant non-syndromic microtia and distinctive ear morphology”. *Clin Genet*. 91(5):774-779.

Pinson L, Perrin A, Plouzennec C, Parent P, Metz C, Collet MJ, Le Bris MJ, Douet-Guilbert N, Morel F, De Braekeleer M. (2005). “Detection of an unexpected subtelomeric 15q26.2--> qter deletion in a little girl: clinical and cytogenetic studies”. *Am J Med Genet A*.138:160–165.

Poswillo D. (1974) “Otomandibular deformity: pathogenesis as a guide to reconstruction”. *J Maxillofac Surg*. 2:64–72.

Poswillo D.(1975) “The pathogenesis of the Treacher Collins syndrome (mandibulofacial dysostosis)”. *Br J Oral Surg*. 13:1–26.

Potocki L, Bi W, Treadwell-Deering D, Carvalho CM, Eifert A, Friedman EM, Glaze D, Krull K, Lee JA, Lewis RA, Mendoza-Londono R, Robbins-Furman P, Shaw C, Shi X, Weissenberger G, Withers M, Yatsenko SA, Zackai EH, Stankiewicz P, Lupski JR. (2007). “Characterization of Potocki-Lupski syndrome (dup(17)(p11.2p11.2)) and delineation of a dosage-sensitive critical interval that can convey an autism phenotype.” *Am J Hum Genet*. 80(4):633-49.

Qiu M, Bulfone A, Ghattas I, Meneses JJ, Christensen L, Sharpe PT, Presley R, Pedersen RA, Rubenstein JL. (1997) “Role of the Dlx homeobox genes in proximodistal patterning of the branchial arches: mutations of Dlx-1, Dlx-2, and Dlx-1 and -2 alter morphogenesis of proximal skeletal and soft tissue structures derived from the first and second arches”. *Dev. Biol*. 185, pp. 165–184.

Qiu M, Bulfone A, Martinez S, Meneses JJ, Shimamura K, Pedersen RA, Rubenstein JL. (1995) “Null mutation of *Dlx-2* results in abnormal morphogenesis of proximal first and second branchial arch derivatives and abnormal differentiation in the forebrain”. *Genes Dev*. 9, pp. 2523–2538.

Quintero-Rivera F, Martinez-Agosto JA. (2013) "Hemifacial microsomia in cat-eye syndrome: 22q11.1-q11.21 as candidate loci for facial symmetry". *Am J Med Genet A*. 161A:1985– 1991.

Redon R, Ishikawa S, Fitch KR, Feuk L, Perry GH, Andrews TD, Fiegler H, Shapero MH, Carson AR, Chen W, Cho EK, Dallaire S, Freeman JL, González JR, Gratacòs M, Huang J, Kalaitzopoulos D, Komura D, MacDonald JR, Marshall CR, Mei R, Montgomery L, Nishimura K, Okamura K, Shen F, Somerville MJ, Tchinda J, Valsesia A, Woodwark C, Yang F, Zhang J, Zerjal T, Zhang J, Armengol L, Conrad DF, Estivill X, Tyler-Smith C, Carter NP, Aburatani H, Lee C, Jones KW, Scherer SW, Hurles ME. (2006) "Global variation in copy number in the human genome" *Nature*. 444(7118):444-54.

Richman JM, Lee SH (2003). "About face: signals and genes controlling jaw patterning and identity in vertebrates". *Bioessays*. 25:554–568

Rieder MJ, Green GE, Park SS, Stamper BD, Gordon CT, Johnson JM, Cunniff CM, Smith JD, Emery SB, Lyonnet S, Amiel J, Holder M, Heggie AA, Bamshad MJ, Nickerson DA, Cox TC, Hing AV, Horst JA, Cunningham ML. (2012). "A human homeotic transformation resulting from mutations in PLCB4 and GNAI3 causes auriculocondylar syndrome". *Am J Hum Genet.* 90:907–914.

Ritchey ML, Norbeck J, Huang C, Keating MA, Bloom DA. (1994) "Urologic manifestations of Goldenhar syndrome". *Urology*.43(1):88-91.

Rivera-Perez J.A, Mallo M, Gendron-Maguire M, Gridley T, Behringer T. (1995) "Goosecoid is not an essential component of the mouse gastrula organizer but is required for craniofacial and rib development". *Development*. 121 pp. 3005–3012

Roback EW, Barakat AJ, Dev VG, Mbikay M, Chretien M, Butler MG. (1991) "An infant with deletion of the distal long arm of chromosome 15 (q26.1----qter) and loss of insulin-like growth factor 1 receptor gene". *Am J Med Genet*. 38:74–79.

Roca C, Adams RH. (2007). "Regulation of vascular morphogenesis by Notch signaling". *Genes Dev.*21:2511–2524.

Rollnick BR, Kaye CI, Nagatoshi K, Hauck W, Martin AO. (1987) "Oculoauriculovertebral dysplasia and variants: phenotypic characteristics of 294 patients." *Am J Med Genet*. 26(2):361-75.

Rollnick BR, Kaye CI. (1983) "Hemifacial microsomia and variants: pedigree data."*Am J Med Genet*.15(2):233-53.

Rollnick BR.(1988) " Oculoauriculovertebral anomaly: variability and causal heterogeneity". *Am J Med Genet Suppl*. 4:41–53.

Rooryck C, Souakri N, Cailley D, Bouron J, Goizet C, Delrue MA, Marlin S, Lacombe FD, Arveiler B. (2010) "Array-CGH analysis of a cohort of 86 patients with oculoauriculovertebral spectrum". *Am J Med Genet A*.152A(8):1984-9.

Rosa RF, Graziadio C, Lenhardt R, Alves RP, Paskulin GA, Zen PR. (2010) "Central nervous system abnormalities in patients with oculoauriculo-vertebral spectrum (Goldenhar syndrome)". *Arq Neuropsiquiatr*. 68:98–102.

Sethi MK, Buettner FF, Ashikov A, Krylov VB, Takeuchi H, Nifantiev NE, Haltiwanger RS, Gerardy-Schahn R, Bakker H. (2012) "Molecular cloning of a xylosyltransferase that transfers the second xylose to O-glucosylated epidermal growth factor repeats of notch". *J Biol Chem.* 287:2739–2748.

Shaffer LG, Lupski JR. (2000) "Molecular mechanisms for constitutional chromosomal rearrangements in humans". *Annu Rev Genet*. 34:297-329.

Sim SC, Risinger C, Dahl ML, Aklillu E, Christensen M, Bertilsson L, Ingelman-Sundberg M. (2006) "A common novel CYP2C19 gene variant causes ultrarapid drug metabolism relevant for the drug response to proton pump inhibitors and antidepressants". *Clin Pharmacol Ther*. 79(1):103–113.

Smith RJH. "Branchiootorenal Spectrum Disorders".In: Pagon RA, Adam MP, Ardinger HH, Wallace SE, Amemiya A, Bean LJH, Bird TD, Ledbetter N, Mefford HC, Smith RJH, Stephens K, editors. GeneReviews® [Internet]. Seattle (WA): University of Washington, Seattle; 1993-2017.1999 Mar 19 [updated 2015 Oct 22].

Storm AL, Johnson JM, Lammer E, Green GE, Cunniff C.(2005) "Auriculo-condylar syndrome is associated with highly variable ear and mandibular defects in multiple kindreds". *Am J Med Genet A.* 138A:141–5.

Strömland K, Miller M, Sjögreen L, Johansson M, Joelsson BM, Billstedt E, Gillberg C, Danielsson S, Jacobsson C, Andersson-Norinder J, Granström G. (2007) "Oculo-auriculovertebral spectrum: associated anomalies, functional deficits and possible developmental risk factors". *Am J Med Genet A*. 143: 1317–1325

Sullivan-Klose TH, Ghanayem BI, Bell DA, Zhang ZY, Kaminsky LS, Shenfield GM, Miners JO, Birkett DJ, Goldstein JA. (1996) "The role of the CYP2C9-Leu359 allelic variant in the tolbutamide polymorphism". *Pharmacogenetics*. 6(4):341–349.

Szabo-Rogers HL, Smithers LE, Yakob W, Liu KJ.(2010) "New directions in craniofacial morphogenesis". *Dev Biol*. 341:84–94.

Tan TY, Collins A, James PA, McGillivray G, Stark Z, Gordon CT, Leventer RJ, Pope K, Forbes R, Crolla JA, Ganesamoorthy D, Burgess T, Bruno DL, Slater HR, Farlie PG, Amor DJ. (2011) "Phenotypic variability of distal 22q11.2 copy number abnormalities". *Am J Med Genet*.155A(7):1623-33.

Tasse C, Böhringer S, Fischer S, Lüdecke HJ, Albrecht B, Horn D, Janecke A, Kling R, König R, Lorenz B, Majewski F, Maeyens E, Meinecke P, Mitulla B, Mohr C, Preischl M, Umstadt H, Kohlhase J, Gillessen-Kaesbach G, Wieczorek D. (2005) "Oculo-auriculo-vertebral spectrum (OAVS): clinical evaluation and severity scoring of 53 patients and proposal for a new classification". *Eur J Med Genet*. 48(4):397-411.

Tasse C, Majewski F, Böhringer S, Fischer S, Lüdecke HJ, Gillessen-Kaesbach G, Wieczorek D. (2007) "A family with autosomal dominant oculo-auriculo-vertebral spectrum". *Clin Dysmorphol*.16(1):1-7.

Thommen L, Bubl R, Fliegel CP. (1986) "Lipoma of the corpus callosum as a constituent of Goldenhar syndrome". *Monatsschr Kinderheilkd*. 134:541–543.

Torti EE, Braddock SR, Bernreuter K, Batanian JR (2013) "Oculo-auriculo-vertebral spectrum, cat eye, and distal 22q11 microdeletion syndromes: a unique double rearrangement."*Am J Med Genet*. 161A(8):1992-8.

Trainor PA, Dixon J, Dixon MJ (2009) "Treacher Collins syndrome: etiology, pathogenesis and prevention". *Eur J Hum Genet.* 17: 275–283.

Tribioli C, Lufkin T. (1999) "The murine Bapx1 homeobox gene plays a critical role in embryonic development of the axial skeleton and spleen". *Development.* 126:5699–5711.

Trumpp A, Depew MJ, Rubenstein JLR, Bishop JM, Martin GR. (1999) "Cre-mediated gene inactivation demonstrates that FGF8 is required for cell survival and patterning of the first branchial arch". *Genes Dev*. 13(23): 3136–3148.

Tsai FJ, Tsai CH.(1993) "Autosomal dominant inherited oculo-auriculo-vertebral spectrum: report of one family". *Zhonghua Min Guo Xiao Er Ke Yi Xue Hui Za Zhi*. 34(1):27-31.

Tucker AS, Yamada G, Grigoriou M, Pachnis V, Sharpe PT. (1999) "Fgf-8 determines rostral-caudal polarity in the first branchial arch". *Development.* 126:51–61.

Uuspää V.(1978) “Combined bilateral external ear deformity and hypoplastic mandible”. Case report. *Scand. J. Plast. Reconstr. Surg*. 12:165–167.

Vendramini-Pittoli S, Kokitsu-Nakata NM. (2009). “Oculoauriculovertebral spectrum: Report of nine familial cases with evidence of autosomal dominant inheritance and review of the literature”. *Clin Dysmorphol*. 18:67–77.

Vento A.R, LaBrie R.A, Mulliken J.B. (1991) “The O.M.E.N.S. classification of hemifacial microsomia”. *Cleft Palate Craniofac. J*. 28 (1) pp. 68–777

Vincent M, Geneviève D, Ostertag A, Marlin S, Lacombe D, Martin-Coignard D, Coubes C, David A, Lyonnet S, Vilain C, Dieux-Coeslier A, Manouvrier S, Isidor B, Jacquemont ML, Julia S, Layet V, Naudion S, Odent S, Pasquier L, Pelras S, Philip N, Pierquin G, Prieur F, Aboussair N, Attie-Bitach T, Baujat G, Blanchet P, Blanchet C, Dollfus H, Doray B, Schaefer E, Edery P, Giuliano F, Goldenberg A, Goizet C, Guichet A, Herlin C, Lambert L, Leheup B, Martinovic J, Mercier S, Mignot C, Moutard ML, Perez MJ, Pinson L, Puechberty J, Willems M, Randrianaivo H, Szakszon K, Toutain A, Verloes A, Vigneron J, Sanchez E, Sarda P, Laplanche JL, Collet C. (2016) “Treacher Collins syndrome: a clinical and molecular study based on a large series of patients”. *Genet Med*. 18:49–56.

Vissers LE, Veltman JA, van Kessel AG, Brunner HG.(2005) “ Identification of disease genes by whole genome CGH arrays”. *Hum Mol Genet.* 15;14 215-23.

Voigt C, Mégarbané A, Neveling K, Czeschik JC, Albrecht B, Callewaert B, von Deimling F, Hehr A, Falkenberg Smel M, König R, Kuechler A, Marcelis C, Puiu M, Reardon W, Riise Stensland HM, Schweiger B, Steehouwer M, Teller C, Martin M, Rahmann S, Hehr U, Brunner HG, Lüdecke HJ, Wieczorek D.(2013) “Oto -facial syndrome and esophageal atresia, intellectual disability and zygomatic anomalies—expanding the phenotypes associated with EFTUD2 mutations”. *Orphanet J Rare Dis*. 8:110.

Wang JC1, Khan A.(2010) “Large distal 5p deletion with hemifacial microsomia and absence of cri-du-chat syndrome”. *Clin Dysmorphol*.19(1):38-9.

Wang R, Martínez-Frías ML, Graham JM. (2002) “Infants of diabetic mothers are at increased risk for the oculo-auriculo-vertebral sequence: A case-based and case-control approach”. *J. Pediatrics*. Volume 141, Issue 5, Pages 611–617.

Wang S, Zhang J, Zhao A, Hipkens S, Magnuson MA, Gu G (2007). “Loss of Myt1 function partially compromises endocrine islet cell differentiation and pancreatic physiological function in the mouse”. *Mech Dev*. 124(11-12):898-910.

Webler AC, Michaelis UR, Popp R, Barbosa-Sicard E, Murugan A, Falck JR, Fisslthaler B, Fleming I.(2008) "Epoxyeicosatrienoic acids are part of the VEGF-activated signaling cascade leading to angiogenesis". *Am J Physiol Cell Physiol*. 295(5):C1292–1301.

Wieczorek D, Gener B, González MJ, Seland S, Fischer S, Hehr U, Kuechler A, Hoefsloot LH, de Leeuw N, Gillessen-Kaesbach G, Lohmann DR.(2009) "Microcephaly, microtia, preauricular tags, choanal atresia and developmental delay in three unrelated patients: a mandibulofacial dysostosis distinct from Treacher Collins syndrome". *Am J Med Genet A*. 149A (5): 837-843.

Wise CA, Chiang LC, Paznekas WA, Sharma M, Musy MM, Ashley JA, Lovett M, Jabs EW.(1997) "TCOF1 gene encodes a putative nucleolar phosphoprotein that exhibits mutations in Treacher Collins Syndrome throughout its coding region". *Proc Natl Acad Sci U S A*. 94: 3110–3115.

Wiszniewska J, Bi W, Shaw C, Stankiewicz P, Kang SH, Pursley AN, Lalani S, Hixson P, Gambin T, Tsai CH, Bock HG, Descartes M, Probst FJ, Scaglia F, Beaudet AL, Lupski JR, Eng C, Cheung SW, Bacino C, Patel A.(2014) "Combined array CGH plus SNP genome analyses in a single assay for optimized clinical testing". *Eur J Hum Genet*. 22(1):79-87.

Xu J, Fan YS, Siu VM. (2008) "A child with features of Goldenhar syndrome and a novel 1.12 Mb deletion in 22q11.2 by cytogenetics and oligonucleotide array CGH: is this a candidate region for the syndrome?". *Am J Med Genet*.146A(14):1886-9.

Yamada G, Mansouri A, Torres M, Stuart ET, Blum M, Schultz M, De Robertis EM, Gruss P. (1995) "Targeted mutation of the murine goosecoid gene results in craniofacial defects and neonatal death". *Development*. 121 pp. 2917–2922.

Yao Y, Yamamoto K, Nishi Y, Nogi Y, Muramatsu M. (1996) "Mouse RNA polymerase I 16-kDa subunit able to associate with 40-kDa subunit is a homolog of yeast AC19 subunit of RNA polymerases I and III". *J Biol Chem*. 271:32881–32885.

Yasuda K, Morihana E, Fusazaki N, Ishikawa S. (2016) "Cardiovascular Malformations in CHARGE Syndrome with DiGeorge Phenotype: Two Case Reports". *Case Rep Pediatr.* 8013530.

Yokoyama A, Igarashi K, Sato T, Takagi K, Otsuka I M, Shishido Y, Baba T, Ito R, Kanno J7, Ohkawa Y, Morohashi K, Sugawara A. (2014) "Identification of myelin transcription factor 1 (MyT1) as a subunit of the neural cell type-specific lysine-specific demethylase 1 (LSD1) complex". *J Biol Chem*. 289(26):18152-62.

Zhu L, Peng JL, Harutyunyan KG, Garcia MD, Justice MJ, Belmont JW. (2007) "Craniofacial, skeletal, and cardiac defects associated with altered embryonic murine Zic3 expression following targeted insertion of a PGK-NEO cassette." *Front Biosci*. 12:1680-1690.