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Intersections of Epigenetics, Twinning and Developmental Asymmetries: Insights Into Monogenic and Complex Diseases and a Role for 3D Facial Analysis

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For decades the relationships of twinning and alterations in body patterning, such as laterality and asymmetry, have been investigated. However, the tools to define and quantify these relationships have been limited and the majority of these studies have relied on associations with subjectively defined phenotypes. The emerging technologies of 3-dimensional (3D) facial scanning and geometric morphometrics are providing the means to establish objective criteria, including measures of asymmetry, which can be used for phenotypic classification and investigations. Additionally, advances in molecular epigenetics provide new opportunities for novel investigations of mechanisms central to early developmental processes, twinning and related phenotypes. We review the evidence for overlapping etiologies of twinning, asymmetry and selected monogenic and complex diseases, and we suggest that the combination of epigenetic investigations with detailed and objective phenotyping, utilizing 3D facial analysis tools, can reveal insights into the genesis of these phenomena.

■ **Keywords:** twins, asymmetry, epigenetics, 3-dimensional facial scanning

Facial growth is subject to asymmetry, which may be directional or fluctuating. Directional asymmetry occurs when an anatomical character is systematically greater on one side than the other, as compared to fluctuating asymmetry where there are random deviations in symmetry of character traits (Van Valen, 1962). It is suggested that the degree and/or pattern of asymmetry that may be of most interest and this is considered to be reflective of vitality (Jones, 1987) and/or pathology (DeLeon, 2007).

Twinning and alterations in body patterning such as asymmetry (Boklage, 1987), laterality disturbance (Steinman, 2001) and mirror-image twins (Wszelaki, 1953) are of significant scientific interest and some clinical reports provide evidence for an interwoven genesis of these phenomena (Thacker et al., 2009).

For at least 60 years the relationships between these entities, mainly predicated on data from animal studies undertaken in the first half of the 20th century, have been investigated (Torgersen, 1950). However, the tools to define and quantify these relationships have been limited and, with some exceptions (Brown et al., 1987; Townsend et al., 1986), the majority of these studies have relied on associations with subjective clinically defined phenotypes.

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Advances in molecular genetics have provided new opportunities for novel investigations of mechanisms central to early developmental processes, twinning and related phenotypes. Epigenetics, the study of mitotically or meiotically heritable changes in expression without changes in DNA sequence, is expanding our knowledge of developmental biology and disease (Feinberg, 2010; Groom et al., 2010). One of the major epigenetic mechanisms involves DNA methylation, which can modulate genetic imprinting, the phenomenon by which certain genes are expressed in a parent-of-origin-specific manner (Callinan & Feinberg, 2006). This occurs at imprinting control regions (ICRs), which act as discrete cis-acting DNA elements within clusters of imprinted genes, with methylation state inherited through the germline. Imprinting errors have been recently implicated in developmental asymmetry (Bestor, 2003) and interrelationships of these developmental phenomena with monogenic (Bliek et al., 2009) and complex (Feinberg, 2010) diseases have been established.

Complementary to these developments are the emerging technologies of 3-dimensional (3D) facial scanning and geometric morphometrics that are providing the means to establish objective criteria (Smeets et al., 2010), including measures of asymmetry that can be used for phenotypic classification. We review evidence for overlapping etiologies of twinning, asymmetry and selected monogenic and complex diseases. It is suggested that the combination of (epi)genetic investigations with detailed and objective phenotyping, utilizing emerging 3D facial analysis tools, can reveal insights into the pathogenesis of these phenomena.

Before directly addressing observations suggesting the intersection of the above phenomena, a brief overview of factors impacting twinning is appropriate.

Secular Changes in Rates of Twinning and Implications for Disease

There has been an approximate doubling of the rate of twin births recorded in the United States over the past two decades (Martin et al., 2006), with similar patterns across the developed world (Blondel & Kaminski, 2002). There are geographic variations in twin rates within the United States (CDC, 1997), but the most significant correlate is with advanced maternal age (AMA; CDC, 1997), which accounts for 1/3 of the increase in twin pregnancies internationally (Blondel & Kaminski, 2002). Another factor is assisted reproduction technology (ART) (Wright et al., 2008). Commensurate with these increased rates are disproportionate morbidities compared to singletons, in particular prematurity (March of Dimes, 2008) and congenital anomalies (Firth et al., 2005). There are implications for the prevalence of monogenic diseases (Bliek et al., 2009), and complex phenotypes have also been implicated. The clarification of factors impacting on or associated with twinning is a health imperative.

Factors Impacting on Twinning

Mechanisms underlying twinning are both complex and multifactorial with interactions of environmental and genetic factors, which may be mediated via epigenetic mechanisms. They can be linked to zygosity, although there is some overlap. Those associated with dizygotic (DZ) twinning can be considered as maternal factors and include AMA and ART; maternal body mass index and height are also implicated (Firth et al., 2005). There are familial and ethnic relationships with DZ twinning frequency, for example Nigerians have a relatively high rate of 1:11 compared to the Japanese at 1:250 (Leszczynska-Gorzelak et al., 2000). The high rate in Nigerians has been postulated to be an environmental influence of elevated estrogen with yam consumption coupled with high dairy intake modulating insulin growth factor 1 (IGF-1) (Steinman, 2006).

Familial (Machin, 2009) and ART factors are also implicated in monozygotic (MZ) twinning. Of particular interest are the (epi)genetic conditions associated with the increased rate of MZ twining, for example Beckwith-Wiedemann (BWS) (Weksberg et al., 2002).

The genetic and environmental factors implicated in twinning may be modified by epigenetic factors. Understanding the factors mediating, or mediated by, the above are important to the understanding of biology and disease.

The Intersection of Asymmetry and Twinning

DISORDERS OF THE CHROMOSOME 11P15 REGION

Beckwith-Wiedemann (BWS)

BWS is an overgrowth condition that can manifest with asymmetric growth of the soma, paired organs and the face. BWS can result from loss of imprinting at two ICRs within chromosome 11p15.5. ICR1 controls the expression of the *IGF2* gene and the non-coding RNA *H19* and is methylated on the paternal allele. ICR2 controls the expression of the *CDKN1C* gene and the antisense RNA *KCNQ1OT1* and is methylated on the maternal allele. Generally, twin pairs with ICR2 imprinting defects are discordant, female and the underlying defect is hypomethylation of the maternal allele. The excess of MZ twins among BWS patients with ICR2 hypomethylation is suggestive of a relationship between a methylation defect and the twinning process. It was first proposed that unequal inner cell mass division, as a consequence of twinning, leads to a differential maintenance of imprinting of the respective twins, where particularly *KCNQ1OT1* is vulnerable to a loss of imprinting event (Weksberg, et al., 2002). Perhaps more likely is the alternative hypothesis that a lack of maintenance of DNA methylation on the maternal allele of ICR2 at a critical stage of preimplanta-

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tion development results in the subsequent loss of imprinting at ICR2 in a proportion of blastomeres within the inner cell mass of the blastocyst. This would lead to epigenetic and functional asymmetry within the blastocyst with blastomeres losing methylation at ICR2 growing faster, leading to growth asymmetry between different cell populations in the inner cell mass, which would trigger the twinning event and lead to MZ twins discordant for BWS (Bestor, 2003). This schema gave rise to the concept of an 'imprinted twinning gene'. Furthermore, it has been suggested that singletons with methylation disturbance disorders may in reality be a product of a twinning event where the sibling has been resorbed (Bliek, et al., 2009) in accordance with the vanishing twin hypothesis (Landy & Keith, 1998).

There is an increased risk of BWS with ART (Sutcliffe et al., 2006) and it is noteworthy that oocytes derived from hormonally stimulated IVF cycles have been shown to demonstrate an aberrant methylation of KCNQ1OT1 (Khoueiry et al., 2008). If there is pathogenetic overlap of ART, BWS and MZ twinning, methylation abnormalities associated with these phenomena may not be limited to the 11p15 region. A subset of BWS MZ twins, and singletons, with DMR2 hypomethylation have aberrant methylation at multiple imprinted loci (Bliek, et al., 2009) and aberrant methylation occurs outside the 11p15 region in some BWS individuals conceived by ART (Rossignol et al., 2006).

Russell-Silver Syndrome (RSS)

Russell-Silver Syndrome (RSS) is also possibly phenomenologically associated with asymmetry and twinning. Subjects with RSS present with short stature and can also manifest with hemihypotrophy of the soma and/ or face. A subset of this condition is associated with disturbances within 11p15.5 ICR1. It can be considered clinically and (epi)genetically as reciprocal to BWS (Eggermann, 2009). MZ twins discordant for RSS have been reported (Begemann et al., 2010; Bergsma et al., 1969; Gicquel et al., 2005; Nyhan & Sakati, 1976; Sagot et al., 1996; Samn et al., 1990; Yamazawa et al., 2008) including three sets of twins in which methylation analysis revealed an epimutation in ICR1 only in the affected twin (Begemann, et al., 2010). Affected co-twins from two of these MZ pairs also showed maternal hypomethylation at ICR2 (Begemann, et al., 2010; Gicquel, et al., 2005). RSS was also described in a child conceived by IVF and who had aberrant methylation of the imprinted gene PEG1/ MEST (Kagami et al., 2007), suggesting that the RSS phenotype may be associated with aberrant methylation outside of the 11p15.5 region.

Other disorders associated with aberrant imprinting may also be involved in the nexus of twinning, ART and asymmetry, as studies suggest an association of ART with other monogenic disorders associated with aberrant imprinting which does not primarily affect the 11p15 region (reviewed in (Manipalviratn et al., 2009).

OCULO-AURICOLO- VERTEBRAL SPECTRUM (OAVS), FACIAL ASYMMETRY, TWINNING, ART AND REPRODUCTION ABNORMALITIES.

The most distinctive group of syndromes presenting with facial asymmetries are the disorders that encompass the Oculo-Auriculo-Vertebral Spectrum (OAVS). This spectrum includes conditions with phenotypic overlaps that predominantly involve derivatives of the first and second branchial arches. Their manifestations are highly variable and those presenting with the most evident facial asymmetry are individuals with the hemifacial microsomia phenotype. This varied presentation is indicative of a causal heterogeneity where a number of environmental, genetic and multifactorial associations have been reported (Genereviews). As in the previous conditions, there are also associations with (epi)genetic mechanisms, multiple pregnancies and ART.

OAVS has been recurrently associated with multiple gestations, predominantly in discordant MZ twins (reviewed in Baynam & Goldblatt, 2009). The incidence of multiple pregnancies is almost 10 times more common in association with OAVS than observed in controls (Lawson et al., 2002). There is also an elevated prevalence of twin pregnancies in cases, which is also observed in family members of index cases, when compared to population prevalence (Werler et al., 2004). Furthermore, there is an excess of malformations, including OAVS, in MZ twins and this has lead to the conclusion of a common cause for both some malformations and MZ twinning (Schinzel et al., 1979).

The concept of interrelationships of these phenomena is further supported by considering spontaneous fetal losses. At least 12% of natural conceptions involve multiple pregnancies, with a considerable attrition rate with only 2% surviving to term as twins and a further 12% resulting in single births (Boklage, 1990). Most twins are lost very early in pregnancy and at least two thirds of twins evident at 10 weeks of gestation are singleton by the time of birth (Levi, 1976). Theoretically, the remaining singletons are at risk for structural congenital anomalies, including OAVS. Some singletons born with anomalies are likely to have been a sibling of a MZ twin (Hall, 2003). In this context, it is noteworthy that OAVS has been reported in a child whose monozygotic twin died in utero (Parisi et al., 1983), and bleeding during the first trimester has been associated with singletons affected with OAVS or microtia (which may be a minimal manifestation of OAVS) (Parisi, et al., 1983). Mechanistically, given shared placental circulations of some MZ twins sharing the same placenta, vascular disturbances such as transient hemodynamic inequalities, embolic phenomena or hemostatic defects have been suggested to render the twin more liable to dysmorphic development (Boles et al., 1987). Therefore, one twin might manifest OAVS and the other be phenotypically normal or one twin might abort and the other manifest OAVS. An alternative or complementary explanation is that OAVS and twinning may share common (epi)genetic origins.

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The association of OAVS and MZ twinning with ART has been extensively reviewed (Baynam & Goldblatt, 2009; Wieczorek et al., 2007). Wieczorek et al. (Wieczorek, et al., 2007) found associations of reproduction abnormalities and twinning in parents of OAVS affected individuals. Their findings included an increased incidence of OAVS in children conceived by ART and an increased frequency of twins amongst OAVS affected children. They noted the parallel of these associations with BWS (Wieczorek, et al., 2007). In accordance with these proposed parallels, KCNQ1OT1 methylation abnormalities have been identified in oocytes from hormonally stimulated cycles (Khoueiry et al., 2008).

The cause(s) of MZ twinning in IVF continue to be a topic of intense investigation. Proposed mechanisms include overripeness ovopathy and intercellular blastocyst discordance. The former postulate is predicated on a frog egg experimental model where an 'overripeness' is positively correlated with monozygotic twining and associated anomalies (Witschi, 1952). Jongbloet described this phenomena as an over-ripeness ovopathy (Jongbloet, 1969) that was conceived of as a 'delay of either fertilization or ovulation … [that results in a] … continuum of reproductive casualties including dysplasias of one or more developmental fields'. He suggested this as a cause for OAVS in the setting of 'high risk conceptions' including in-vitro fertilization (IVF) (Jongbloet, 1987). Given the maturity of oocytes is influenced by the reproductive performance of women and ovarian stimulation procedures (Horsthemke & Ludwig, 2005; Sato et al., 2006), these factors may contribute to MZ twinning and associated phenomena. The latter postulate suggests that discordant cells within the blastocyst, arising from processes including chromosomal anomaly, mutation or epimutation (including methylation disturbance) may identify each other as immunologically foreign resulting in inner cell mass separation (Hall, 2003; James, 2002). Notably, ART Is associated with altered methylation (Manipalviratn, et al., 2009) and there is evidence for epigenetic disturbances of the *BAPX1* gene in OAVS (Fischer et al., 2006).

TURNER SYNDROME, TWINNING, MOSAICISM AND CHIMERISM

Turner's original publication described a child with Turner syndrome (TS) who had a normal twin sister (Turner, 1938), and subsequently there have been many reports of this condition among twin pairs (Nance & Uchida, 1964) and an increased incidence of twins is supported by some studies (Carothers et al., 1980). Furthermore TS, like OAVS, is another condition in which MZ twinning has been found to be more frequent in family members of index cases (Nance & Uchida, 1964). The majority of TS cases have a 45, X chromosome constitution with a related set of mosaic karyotypes including 45,X/ 46,XX mosaicism (Gardner & Sutherland, 2004). A minority of TS individuals are mosaic for a Y chromosome cell line (karyotypically this is 45,X,/46,XY mosaicism; Mendes et al., 1999). There is a wide spectrum of phenotypes associated with 45,X/ 46,XY mosaicism including TS, mixed gonadal dysgenesis and apparently normal males (Telvi et al., 1999). Chromosomal mosaicism is the presence, within the one conceptus, of two or more cell lines that are genetically identical except for the chromosomal difference between them. It is distinguished from chimerism which is the coexistence of more than one cell line in an individual that is due to the union of two originally separate conceptions (Gardner & Sutherland, 2004); distinguishing mosaicism from chimerism can be challenging. A number of pairs of MZ twins in which one or both twins has 45, X/46,XY mosaicism have been reported (reviewed in (Boles, et al., 1987; Costa et al., 1998)). Additionally, there are reports of MZ twins concordant for 45,X/46,XY mosaicism that were discordant for phenotypic sex (Gantt et al., 1980), and chimeric MZ twins with a 45,X/46,XY karyotype have also been identified (Gonsoulin et al., 1990).

Other aspects of mosaicism of interest include that a child with Turner syndrome, somatic asymmetry and 45,X/ 46,XX mosaicism was described to have an RSS phenotype, and a further individual with a female phenotype had a male peripheral blood karyotype (46,XY) and an epimutation at 11p15 in her lymphocytes (Bartholdi et al., 2009). Factors relating to chromosomal mosaicism may also be of importance to epigenetic regulation. Somatic and facial asymmetry are phenotypic hallmarks of chromosomal mosaicism (Possum_dysmorphology_database); however, to the best of the authors' knowledge, asymmetry has not been systematically investigated in Turner syndrome.

3D Facial Analysis: Objective Phenotyping

The capacity to discriminate facial anomalies is critical in the identification of abnormal form associated with many conditions with craniofacial manifestations. Currently this is reliant on subjective assessments that are partly dependent on operator experience and open to inter-operator variability. Furthermore, the molecular cause for many conditions remains to be elucidated and therefore accurate definition of phenotype remains crucial to diagnostics and the understanding of the biology affecting facial morphogenesis.

Phenomics (reviewed in (Houle et al., 2010) is the large scale high-dimensional phenotyping that is a natural complement to genetic, and epigenetic, technologies to facilitate advances in biology. Phenomics can be used to investigate explanations at phenotypic and (epi)genotypic levels. Imaging modalities are one of the promising technologies in this field (Houle et al., 2010). Emerging techniques utilizing 3-dimensional (3D) facial scanning and geometric morphometric analysis of scan data are providing objective and automated means to identify dysmorphology and asymmetries. Additionally, studies employing 3D facial analysis are facilitating examination of the cell biology underpinning facial dysmorphism (Tobin et al., 2008). A number of approaches are being investigated (Adams et al., 2004; Cox-Brinkman et al., 2007; Deleon & Richtsmeier, 2009; Hammond et al., 2008;

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Hammond et al., 2001; Hammond et al., 2005; Richtsmeier et al., 2002; Weinberg et al., 2008) and all utilize methodology dependent on the identification of homologous facial components. They can differ in their means of measuring form differences; however, they are universal in defining form anomaly as either a statistical difference to a normative archetype or differences clustered within an identified area of dysmorphology (Hammond, 2007). However, these approaches do not account for 'normal' facial variations that can impact on their discriminatory power.

Recently, Claes et al. have developed a novel approach that uses an 'expanded' archetype that encompasses normal population facial variations to detect facial anomalies (Claes et al., 2011). These tools utilize a standardized anthropometric mask (AM) that is fitted to 3D facial scans. This mask provides a mapped high density set of corresponding quasi-landmark data that facilitates statistical approaches to detect harmonic and/or disharmonic covariance in spatial relationships within the form of the face (Figure 1). These techniques can be used to generate a normal equivalent (NE) that can be defined as a patient-specific normalized reference and is considered as the harmonious counterpart of the dysmorphic/asymmetric face.

These strategies, with some modification, can also be employed to quantify facial asymmetries (Figure 2). A robust superimposition is performed with a remapped 'mirror' to assess facial asymmetry, where dysmorphology is assessed with superimposition of the synthesized NE (Claes et al., in press).

To provide a measure of discrepancy in form, the degree, distribution and locality of quasi-landmark discordances can be calculated and visualized by color histogram mapping of the patient scans (Figures 1 and 2). A summary statistic reports the overall relative significant discordance (RSD) as a measure of percentage of the face affected, while the root mean square error (RMSE) of the distribution of distances between quasi-landmarks provides a measure of severity (Table 1). These measures can be employed to investigate asymmetry in twins and singletons.

Asymmetry and Syndromic and Complex Diseases

A syndrome is a group of signs and symptoms that collectively indicate a particular condition. Facial asymmetry is a recurring theme in a large number of syndromic conditions, for example a search of the Possum dysmorphology database© using the term facial asymmetry yields 239 conditions (Possum_dysmorphology_database). Some of these conditions are known to be associated with epigenetic disorders and others involve genes, such as *FGRF2, TWIST,* and those of the RAS-MAPK network, that are in pathways associated with developmental processes including cell lineage determination, growth and differentiation (Genecards). These pathways may be subject to epigenetic regulation and therefore their phenotypes may overlap with disorders known to result from epigenetic disturbance.

Facial and somatic asymmetries have also been implicated in evolutionary processes and evolution has been suggested to influence the pathogenesis of complex diseases (Le Souef et al., 2000). Phenomics has been suggested as a tool to investigate the (epi)genetic basis of complex diseases (Houle et al., 2010). Therefore, accurate determination of facial asymmetry may provide for novel applications for investigation of complex phenotypes. Notably, facial asymmetries have been implicated in mate selection preferences and mate selection processes influence evolution. In this context, symmetry in women has been established as an attractive trait as rated by men (Lie et al., 2008). A biological underpinning for this observation may be the association of somatic symmetry and female fertility (Jasienska, 2006). From a twin perspective, asymmetry, including facial asymmetry, has been demonstrated in MZ twins (Burke & Healy, 1993; Yager, 1984). Additionally, in subjective comparisons of monozygotic twin pairs, the twin with perceived facial symmetry was rated as the more attractive. This perceived attractiveness was directly related to the magnitude of the asymmetry (Mealey et al., 1999). It is noteworthy that the association of Interleukin-4 (IL-4) gene, amongst other immunoregulatory genes, with complex phenotypes has been suggested

TABLE 1

Overall NE and Asymmetry Scores for Normative Reference Females, a Female With OAVS[1] and Her Unaffected Monozygotic Twin

Assessment	Subject	RSD or RSA (%)		RMSE (mm)	
NE	Reference range	10.6 (1.8 *SD*) [RSD]		0.91 (0.22 *SD*)	
Asymmetry	Reference females	9.53 (1.21 *SD*) [RSA]		0.94 (0.23 *SD*)	
Assessment	Subject	RSD or RSA (%)	*Z* score (*SD*)	RMSE (mm)	Z-score (SD)
NE	OAVS twin	10.17	-0.24	1.12	+0.95
NE	Unaffected twin	9.36	-0.96	0.82	-0.43
Asymmetry	OAVS twin	13.06	+2.92	2.56	+7.04
Asymmetry	Unaffected twin	11.66	+1.76	1.16	+0.96

Note: RMSE scores the overall degree of discordance (e.g. facial disharmony, or asymmetry) in mm, while the RSD/RSA score quantifies the extent of the discordance/asymmetry as a percentage of the face affected. *Z* scores describe the extent of the difference to reference distributions (i.e., reference range).

[1] Oculo-auriculo-vertebral spectrum; [2] Normal equivalent; Root mean square error; Relative significant discordance.

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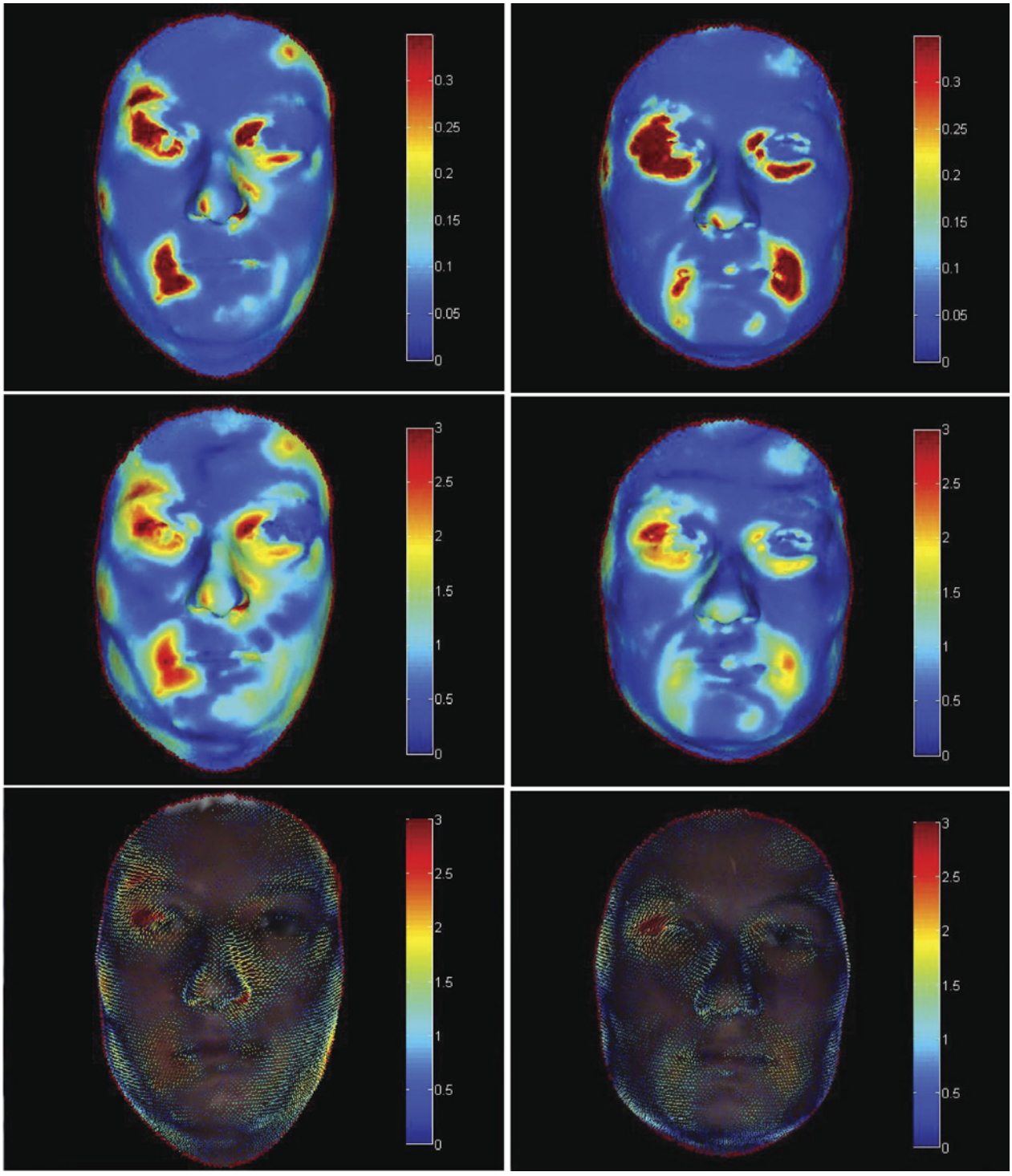

FIGURE 1

Facial normal equivalent assessments of a set of female monozygotic twins discordant for oculo-auriculo-vertebral spectrum (OAVS): affected twin (left column) unaffected twin (right column). Outlier maps (A,B) based on standard deviations highlight areas of discordance, distance maps (C,D) quantify the discordance in mm, and vector maps (E,F) provide directional of the discordance.

to be influenced by evolutionary processes (Baynam et al., 2007; Le Souef, et al., 2000) and epigenetic factors are fundamental to regulation of expression of these genes (Sanders, 2006). Studies of cord blood T cells identified that the most outstanding functional group of IL-4 regulated genes included components of the RAS-MAPK pathway (Lund et al., 2007). Mutations in genes in this pathway occur in Noonan syndrome and related condi-

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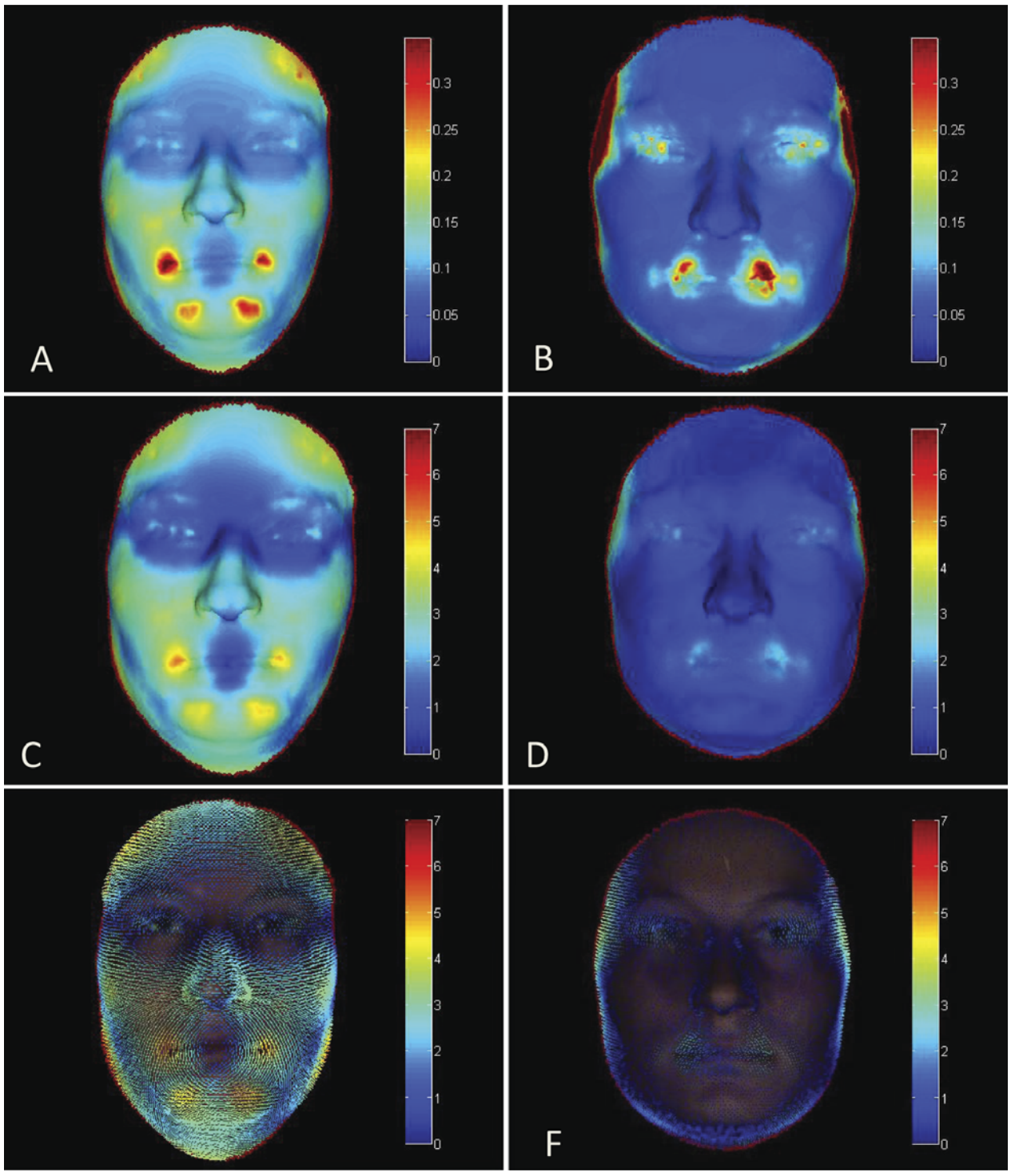

FIGURE 2

Facial asymmetry assessments of a female monozygotic twin pair discordant for OAVS: affected twin (left column), unaffected twin (right column). Outlier maps based on standard deviations measures of the distribution of distances set at 2SD (A,B) highlight areas with the most variance, the distance maps (C,D) quantify the amount of divergence in mm, and the vector maps (E,F) provide additional directional information.

tions (Genereviews) and facial asymmetry is one of the principal modes of facial variance of this condition (Hammond et al., 2004). Finally, a relationship between schizophrenia, asymmetry (of the brain) and twinning has been postulated (Boklage, 1977). These factors suggest interacting networks underlying asymmetries, that have been influenced by evolution, are partly epigenetically regulated and that this may have relevance for complex

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disease. Given the relationships between asymmetry and twinning, these networks may have further relevance to twin studies.

In conclusion, tools for 3D facial analysis may provide the resolution to identify and quantify phenotypic variations, including degrees of facial asymmetry, to facilitate novel investigations of twinning, syndromic and complex diseases. Ultimately, investigations of the networks mediating intersections of these phenomena will require phenomic assessments to be coupled with (epi)genetic studies.

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