Author's response to reviews

Title: Twin discordance and disease: not just an environmental cause?

Authors:

Witold WC Czyz (witold@well.ox.ac.uk)
Julia JMM Morahan (julia.morahan@well.ox.ac.uk)
George GCE Ebers (George.Ebers@clneuro.ox.ac.uk)
Sreeram SVR Ramagopalan (s.ramagopalan@qmul.ac.uk)

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Author's response to reviews: see over
Discordance in monozygotic twins and disease.

Dear Sir/Madam,

We would like to submit the attached manuscript for your consideration for publication as a review article in your journal.

For more than a century, twins have been an important source of information in studying human trait heritability. Since MZ twins have been assumed to be genetically identical, any phenotypic discordance in such twins has often been explained by non-shared environmental factors acting after birth. Recent studies have cast a new light on MZ twin discordance and shown this explanation as being far too simple. We review here other reasons for discordance, including differences in the in utero environment, mosaicism and epigenetics. Starting from environmental factors, the review explores the effects of twin pregnancy on early discordance with a particular attention to the role of chorionicity and amnionicity. Subsequently, de novo mutations and genetic mosaicism are explored and developmental noise and stochastic factors, including differential allelic and random monoallelic gene expression. After a brief outline of molecular epigenetic mechanisms, the review focuses on the evidence for epigenetic causes of twin discordance, following and reviewing most of the published literature epigenetic studies on MZ twins. The final section outlines the main limitations of current epigenetic research, particularly the issue of causality, as well as guidelines for future studies.

Epigenetic differences are gaining increasing recognition. While it is known that in specific cases epigenetic alterations provide a causal factor in disease aetiology, the overall significance of epigenetics in twin discordance as well as its dependence on environmental and genetic factors remains unclear. Several whole-methylome and epigenome profiling studies attempted to investigate temporal methylation changes and methylome heritability, however provided no clear consensus.

Similarly researchers have often investigated epigenetic contribution in human disease focusing on evidence from MZ twins, yet they have not given a definite answer due to limitations in establishing causality.

New guidelines have been recently proposed in response to the limitations troubling the early-stage epigenetic research and studies with larger twin cohorts and improved longitudinal designs trying to address the issue of causality are emerging. This constitutes a promising improvement which has already produced positive results although, unlike GWAS, epigenome-wide studies face other important limitations which will make their implementation and wider use more challenging.
We believe this review will be of great interest to your readership since it explores the subject of twin discordance and epigenetics as an important component in human phenotypic variability.

The authors’ contributions are as following:

**Review design and concepts:** Witold Czyz, Dr Sreeram V Ramagopalan, Professor George Ebers  
**Analysis and Data interpretation:** Witold Czyz,  
**Manuscript draft:** Witold Czyz  
**Critical revision of the manuscript:** Dr Julia Morahan, Dr Sreeram V Ramagopalan, Professor George Ebers  
**Study supervision:** Dr Sreeram V Ramagopalan, Professor George Ebers

All the authors gave their final approval for the submission of the manuscript. The authors of the review can be contacted under the following email addresses:

- gcpebers@gmail.com  
- sreeram@ramagopalan.net  
- witold@well.ox.ac.uk  
- julia.morahan@well.ox.ac.uk

All tables and figures have been created specifically for the review by one of the authors, Witold Czyz. The section below contains the response to the points raised by the reviewers.

**Reviewer 1 - Dr Esteban Ballestar**

The manuscript by Czyz and colleague deals with the interesting topic of monozygotic twin discordance and the increasing recognition of epigenetics as a mechanism to explain this phenomenon. The review is well written and has a wide prospective on the potential factors to explain MZ discordance. It will appeal to a wide audience. My only concern is that the authors oppose environment with epigenetics, even in the title. I do think that environmental factors and epigenetic mechanisms are at different levels and cannot be compared with each other. In fact, a variety of environmental factors are a potential source to generate epigenetic changes (at least in part), and this should be clearer stated in the text. Epigenetic changes can occur as a result of environmental factors, stochastic changes or epimutations and as a result of mutations taking place at genes encoding elements of the epigenetic machinery. In this review the focus is therefore brought from the environmental level to another level, where environment is just another component. The text should be modified to clarify this issue. This change should also affect the title.

- **Reply:** The title has been amended to reflect the fact that epigenetic changes are an effect of different genetic, environmental and stochastic factors shaping phenotypic diversity – rather than another independent factor. This point was further clarified in the Abstract as well as in the “In Utero Environment and Discordance”, “Developmental Noise” and “Stochasticity and Epigenetics” section. Changed and added sentences are listed below:

- Title: **Genetic, environmental and stochastic factors in Monozygotic Twin discordance with a focus on epigenetic differences.**
Abstract: genetic mosaicism and stochastic factors, focusing on epigenetic discordance

Abstract: It is also challenging to determine the causality and relative contributions of environmental, genetic and stochastic factors to epigenetic variability.

Abstract: This review explores the subject of epigenetics as another component in human phenotypic variability as well as its links to disease focusing on evidence from MZ twin studies.

Page 7 and 8: Occasional abnormal cytosine methylation can result in epigenetic alterations called epimutations which, just like DNA mutations, can deactivate or cause both copies of the imprinted gene to be transcriptionally active[75]. Such alterations can be broadly divided into three different categories, depending on their origin. Some epimutations have a direct genetic cause and are secondary to a DNA mutation in cis or in trans, for instance mutations to imprinting centres. Other epimutations are primary with no sequence alteration [75, 80] This latter category could be further divided into stochastic epimutations, caused by the inherently error-susceptible molecular machinery, and epimutations which are environmental in origin. This distinction can be arbitrary since environment can potentially cause stochastic epimutations.

Page 8 and 9: The significance of the environment and genes in driving epigenetic changes is a subject of debate with some authors claiming that epimutations might be stochastic in nature and offer an alternative, non-heritable and non-environmental, explanation for phenotypic variability [2, 72, 73, 82]. The key argument rests on the assumption that the random character of de novo faults in DNA methylation, whose fidelity is estimated to be at the level of 97-99.9% in cell culture but lower in vivo, cannot be ascribed to heritable genetic predispositions nor to the environment [2, 73]. The concept of stochastic epimutations as the third source of variation in opposition to genetic and environmental effects has important limitations and rests on arbitrary assumptions since it is not evident that the random faults in methylation maintenance cannot be themselves genetically determined (in a similar way to DAE), or of environmental origin.

Reviewer 2 - Dr Mario Fraga

This manuscript reviews the role of epigenetics in twin discordance and discusses the relative contribution of a number of possible factors on epigenetic variation over time. Whilst the topic is interesting, the manuscript needs substantial improvement before being published:

The title does not reflect the content of the review because it does not contain the word epigenetics. Moreover, what does ‘twin discordance and disease’ mean? Disease discordance in twins? What do the authors want to suggest by "not just an environmental cause””? On reading the abstract it seems that the alternatives are "differences in the in utero environment, mosaicism and epigenetics". In my opinion, the alternatives to adult environmental conditions are in utero environmental conditions and stochastic molecular alterations (during embryo development and adult life) that depend, at the same time, on genetic and extrinsic factors. These stochastic and
environmental factors can affect the epigenetic marks and generate mosaicism. An alternative title could be "The value of twin studies in environmental epigenetics" or something similar.

- **Reply:** In addition to the changes implemented in reply to points raised by Dr Ballestar, the issue of stochastic epigenetic changes has been clarified in the context of literature in the “In Utero Environment and Discordance”, “Developmental Noise” and “Stochasticity and Epigenetics” sections. The concept of stochastic change as well as the definition of what constitutes a boundary between stochastic and environmental alterations is largely arbitrary and depends on the definition of the environment. It is of course well possible that random faults in methylation maintenance are caused by a combination of genetic and environmental factors, nonetheless their exact cause is not clear and some authors favour the concept of non-environmental and non-genetic stochastic changes and clearly place them on par with environmentally- and genetically-triggered epigenetic changes. Both sides of the argument have been stated in the review.

- **Page 3:** In some studies, similar pre- and postnatal conditions, for example shared in utero environment or upbringing in one family, have been thought to promote phenotypic concordance in contrast to non-shared exposures [2, 3]. However, the concept of non-shared environment has important practical limitations. It is difficult to unambiguously identify the distinct factors and explain their differential effects on phenotype [3].

The concept of non-shared environmental conditions should be clarified. The authors assume that during the embryos’ development the environmental conditions are identical but this is not accurate as it is known that, for example, one of the siblings may receive more nutrients than the other. There are many paragraphs that seem to be out of context and some sections that do not contain what is stated in their title. One example is "Environment and discordance" in which the authors mainly explain the biology of twinning.

- **Reply:** The concepts of shared vs non-shared environment, and in utero environment have been clarified in the “In Utero Environment and Discordance” section, whose title replaced the less accurate “Environment and discordance”. The review now explains that sharing a common uterus or a placenta does not immediately constitute a shared in utero environment. In fact there is reason to believe that multifetal pregnancies often create unequal environment which subsequently leads to phenotypic discordance in monozygotic twins.

- **Page 3:** For instance while MZ twins indeed share a single uterus in multi-foetal pregnancies, they do not necessarily share a common in utero environment.

- **Page 4:** Some of these events like unequal division of blastomeres or uneven vascularisation of the placenta can be considered as non-shared early exposures which can be classified, depending on the adopted definition, as environmental or stochastic.

The paragraph "Slight differences in eye or hair color as ...... from shared environmental exposures in twins." should be rewritten. These phenotypic differences can arise from stochastic and/or environmental-dependent molecular alterations during embryo development and/or adult life but the underlying molecular mechanisms are still largely unknown.
Reply: The paragraph mentioning differences in hair and eye colour in the “Developmental Noise” section has been amended to acknowledge that the underlying mechanisms promoting such cases of discordance are largely unknown.

Page 6: Certain cases of twin discordance might potentially be stochastic in origin, however since the causal mechanisms are not thoroughly understood, it is difficult to separate them from environmental effects and gene-environment interactions. Examples include discordance for eye or hair colour as well as fingerprint profiles, cases of mirror twinning (affecting up to 25% of MZ twins) and major malformations [22].

The paragraph "Certain cases of differential allelic expression (DAE), including random monoallelic ....... within between (sic) the co-twins, still leaving room for stochastic effects." should be rewritten as it contains many factual inaccuracies: Cheung et al found that "in 50% of genes expressed in lymphoblastoid B cells, the entire distribution of the allelic expression ratio is significantly shifted away from the expected mean of 0.5 (equal allelic expression)" and not, as the authors state, that "about 50% of heterozygous loci are subject to DAE within MZ twin pairs"; Baranzini’s study analyzed 3 pairs of twins (and not only a single pair), etc.

Reply: The paragraph on the differential allelic expression (DAE) has been rewritten to match the information given in the reference (Cheung et al.,) and factual errors corrected. Although the Baranzini et al. study did encompass three MZ twin pairs, to the best of our knowledge only one pair was used to estimate the extent of DAE.

Page 6: Certain cases of differential allelic expression (DAE) which result in random monoallelic gene expression arising as a result of X-inactivation or allelic exclusion in olfactory and pheromone receptor genes can constitute a mechanism for stochastically-driven phenotypic discordance in MZ twins[22, 65-70]. Although DAE has been estimated to also affect about 50% of autosomal genes in B-cells, the evidence from MZ twins indicates that the overall degree of DAE is to a certain extent under genetic control with an estimated 30% of the affected genes showing significant correlation between co-twins [71]. The precise estimates of DAE and its concordance in MZ twins vary. In contrast to the previous estimates, a comprehensive whole genome expression experiment conducted by Baranzini et al. (2010) produced indicated that only 1.9% of heterozygous coding loci showed significant evidence for DAE, but out of these, 57% were concordant between the co-twins, still leaving room for stochastic effects[72]. Their findings were however based on a single MZ twin pair [Supplement].

The paragraph "The source as well as significance of epimutations in twinphenotypic variability ....that epimutation rates are lower in cells in vivo[25, 35,80]." should be rewritten. The stochastic epimutations depend on genetic and environmental factors because, for example, the fidelity of the methyltransferases may depend on both genetic and environmental conditions. The authors should also endeavor to clarify the following concept: "there is a stochastic epigenetic variation during embryo development and adult life that depends on genetic and environmental factors but their relative contribution is not known. Taking it into account, the following sentence seems speculative and should be removed:" In contrast to ......showing similar concordance for personality and social attitudes in MZ twins raised apart to those raised together [82].". In
general, I believe that it is difficult to compare the two studies in references 29 and 82 because the age range of the study populations is completely different. In addition, the following inaccuracies should be corrected: The study quoted in reference 29 does not report "a trend of steady accumulating changes to the epigenome with age"; it simply identifies more epigenetic differences in a group of older twins than in another group of younger twins. As the study does not involve a continuous range of ages, it is better not to talk about a tendency.

- **Reply:** In the section on “Epigenetics” the concept of stochastic change has been clarified with a quotation from one study. The sentence mentioning "a trend of steady accumulating changes to the epigenome with age" has been replaced with a more adequate statement referring to two different conditions observed in different age groups rather than a trend.

- Page 9: The extent of epigenetic changes and epigenome heritability is disputable. A thorough cross-sectional study of epigenetic profiles in the lymphocytes of 80 MZ twins, aged between 3 and 74, revealed significantly greater discordance in older individuals [29].

- Page 9: The authors propose that the epigenome is highly heritable at birth, but epimutations arise and accumulate throughout a lifetime and their origin arises due to a combination of external environmental factors and internal “epigenetic drift” arising from defects in methylation. Although such maintenance defects have been claimed by others to represent “endogenous, stochastic mechanisms, independent of environmental perturbations” [83], Fraga et al. do not exclude the notion they might be environmentally-triggered as well.

- Page 10: Kaminsky et al. oppose stochasticity to environmentally induced epigenetic differentiation, favouring the former explanation as the more important in phenotypic discordance of MZ twins[84].

The values in the sentence "Estimates based on 20 MZ and 20 DZ pairs indicated that methylation heritability was very low (0.014) in white blood cells, but rose to about 0.3 in buccal tissue (findings based on 19 MZ and 20 DZ pairs), and up to 0.7 when dichorionic twins only were considered." must be reviewed. They do not correspond with those published in the reference cited.

- **Reply:** The quoted heritability values were calculated from the data given in Kaminsky et al. study as twice the difference in correlation between MZ and DZ twin methylation. However since they have not been given in the text directly, they have been removed.

- Page 9: Estimates based on 20 MZ and 20 DZ pairs indicated that methylation heritability was very low in white blood cells, but rose in buccal tissue (findings based on 19 MZ and 20 DZ pairs), and was significantly greater when dichorionic twins only were considered.

The next paragraph should also be reviewed. In reference 29, young twins present some epigenetic differences but, when compared with older twins these differences are statistically non-significant. The sentence "despite low heritability, the intraclass ...... epigenome-wide findings of ref 29" is not correct because in ref 29, epigenetic differences between twins within the range of ages analyzed in ref 84 (5 and 10-yo) are also stable. In general, the conclusions of the three studies cited in this paragraph are difficult to discuss together for two reasons: Firstly, the technologies used are not the same and this in itself could explain the identification of more
epigenetic differences in young twins in one study than in others and, secondly, because the type of DNA sequences identified in the different studies are not the same (repeated DNA vs single copy genes).

- **Reply**: The paragraph comparing the Wong et al. (2010) and Estellar et al. (2005) studies has been removed since both studies represent two different approaches and vary in the method.
- Page 10: Interestingly, despite low heritability, the intraclass correlation coefficients (ICC) of MZ twins remained stable or increased.

In the section Methylation Studies and Human Disease, the authors should discuss the effect of the tissue-type analyzed. In many of the studies cited, the tissue analyzed is not the disease-targeted tissue. It must be stated that this strategy only allows the identification of systemic epimutations and that tissue-specific epimutations (which, in my opinion are more relevant) are not detected.

- **Reply**: In the “Methylation Studies and Human Disease” section the problem of tissue relevance in epigenetic studies as well as the significance of tissue-specific epimutations has now been mentioned clearly.
- Page 13: Improvements to study designs in the future will likely require sampling from multiple tissues, particularly those that might be relevant to disease as variation of the epigenome varies significantly across different cell types and tissue-specific epimutations may play more important roles than systemic epimutations. However, some tissues are not easily accessible and sampling across different tissues might involve biopsies and post-mortem material [100]. This is an important limitation and some of the recent studies assayed methylation differences in tissues which are not directly relevant to the disease investigated.

Other minor points are: It should be mentioned that Mosaicsisms can also be generated due to epigenetic alterations.

- **Reply**: Epigenetic mosaicism and variegation has been already mentioned in the “Epigenetics” section. This has been expanded on with one more sentence and reference.
- Page 8: Indeed mosaicism in epigenetic alterations has been described in MZ twins and its relevance to phenotype, particularly disease, is the subject of current studies[82].

This sentence needs a reference: "There is evidence for transgenerational inheritance of epigenetic changes but the scope and mechanisms are under study."

- **Reply**: That has been amended.

A paragraph or section should be included which describes studies on environmental epigenetics in genetically identical animals.

- **Reply**: Although we considered adding a section on discordance studies in genetically identical animals and animal epigenetics in the early stage manuscript, we
have decided to limit the scope to human MZ twins only for the sake of coherence and acceptable length of the review.

There many typos and mistakes that must be corrected. Examples are: "were concordant within between the co-twins", "Rather, the term rather described the way", "the significance of age in studies DNA methylation twin discordance", italic format "which have been implicated by a previous study [96]." or the references Esteller et al., 2005, PNAS, Spielman et al., 2008, AJMH).

➢ **Reply:** The text has been re-checked and the typos corrected.

Figure 1 should be improved and clarified.

➢ **Reply:** This has been done.

Thank you very much for your consideration.

We look forward to hearing from you,

Sreeram Ramagopalan MA DPhil
Witold Czyz