

# Preimplantation Genetic Diagnosis for Sex-linked Diseases and Sex-Selection for Non-Medical Reasons

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## Key points

- Sex linked diseases are caused by mutations in genes carried on the X chromosome. Although the penetrance of the trait is typically high in males and low in females caution should be exercised in classifying sex-linked diseases as recessive or dominant.
- Fluorescence in situ hybridization (FISH) is the technique of choice to sex embryos. FISH provides a very robust test with a low risk of misdiagnosis; however, testing that only determines the sex of the embryos cannot discriminate between normal and affected male embryos or between normal and carrier female embryos, which is wasteful of male embryos and accepts a level of risk that carrier females may have clinical features of the trait.
- PCR methodology has been used to develop direct tests for known mutations associated with a range of X-linked diseases. Protocols generally utilise a nested PCR approach, to include one, two or three microsatellite markers closely linked to the disease gene, in order to monitor contamination and allele drop-out, potential sources of misdiagnosis when using PCR-based testing.
- Preimplantation genetic haplotyping (PGH) is a rapid way of developing indirect tests of X-linked disease. The same marker multiplex can be used for all families with the same disease, without the need for the development of family-specific mutation testing. PGH relies on the identification of the chromosome carrying the familial mutation by haplotype analysis of family members. The presence or absence of this chromosome in embryos can then be established by genotyping the biopsied cell. This approach has the added advantage of effectively “fingerprinting” each embryo, which obviates the need for washdrop blanks normally required to monitor contamination.
- Testing for X-linked disease provides special ethical, counselling, and decision-making challenges beyond those posed by autosomal monogenic defects and chromosome rearrangements.
- Many professionals involved in the PGD field take the view that sex selection for a non-medical reason is unacceptable. For others there are varying degrees of ethical risk and the balance between regulation and reproductive freedom is difficult to assess.

## Introduction

Sex-linked diseases are caused by mutations in genes carried on the X chromosome. Typically the penetrance of the sex-linked trait is high in males and low in females; however, the penetrance for some traits can be moderate or high in females. Caution should be exercised in classifying sex-linked diseases to have a recessive or dominant pattern of inheritance as the degree of variability in

heterozygotes is greater than for autosomal traits due to skewed X inactivation, clonal expansion and somatic mosaicism. Online Mendelian Inheritance in Man ([OMIM, 2010](#)) currently lists 1140 entries for genes and/or phenotypes with X-linked inheritance.

X-linked diseases with a recessive pattern of inheritance are the most common. The defective gene on the X chromosome tends to have little effect on heterozygote females because there is a second normal copy of the gene on the other X chromosome. However, males with an X chromosome carrying the defective gene are affected with the disease, as there is no second, normal, X chromosome. The affected male who reproduces will have carrier daughters and normal sons. Relatively common X-linked diseases with a recessive pattern of inheritance are muscular dystrophy and haemophilia A. The muscular dystrophies are a group of hereditary diseases characterized by the progressive loss of muscle cells. Duchenne muscular dystrophy (DMD; [OMIM#310200](#)) is the most common and severe form. Progressive muscle wasting results in loss of mobility and frequently to death as a result of respiratory failure by the late teens or early twenties. Haemophilia A ([OMIM#306700](#)) is an hereditary blood disorder characterized by a deficiency of the Factor VIII blood clotting protein that results in abnormal bleeding; the clinical severity can be mild, moderate or severe.

X-linked diseases with a dominant pattern of inheritance are less common and can be lethal to males in utero. An example is incontinentia pigmenti (IP; [OMIM#308300](#)), which is a disorder that affects the skin, hair, teeth and nails. IP is lethal in most males although there are a few reports of surviving males with a 47,XXY karyotype, somatic mosaicism or different mutations in the IKBKG gene associated with IP.

Sex-linked diseases were the first target of PGD, and the first successful clinical case used PCR to amplify a specific repeat on the Y chromosome to sex embryos in the presence of an X-linked genetic condition (Adrenoleukodystrophy; [OMIM#300100](#)) (Handyside et al., 1990). Failure of the Y chromosome sequence to amplify in some cases led to misdiagnosis (Hardy and Handyside, 1992), and this approach is now therefore no longer recommended. Instead, fluorescence in situ hybridization (FISH)-based PGD was adopted as a more robust technique for the identification of female embryos (Griffin et al., 1994, Munne et al., 1994); however, a small number of centres still use PCR for sexing (Harper et al, 2008). Although most PGD centres use sexing only, many specific mutation analyses for X-linked disease genes have been developed and applied successfully (Sermon et al., 2007, Verlinsky et al., 2002) and the recent introduction of preimplantation genetic haplotyping (PGH) (Renwick et al., 2006) has opened the door to rapid and straightforward development and application of further specific tests for X-linked disease.

## **Approaches to PGD for X-linked diseases**

### *FISH – sexing only, and testing for specific deletion*

The identification of female embryos using FISH is a standard technique in most PGD centres. Commercial probe sets (eg Vysis AneuVysion) are generally used for the centromeres of the X chromosome, Y chromosome and chromosome 18, or another autosome.. The different centromeres are discriminated by the different coloured fluorescent tags associated with the specific DNA probes (see Figure 1). This test is extremely robust as in order to misdiagnose a male chromosome complement as female two errors have to occur: firstly, failure to score the signal for

the Y centromere, and then secondly, to score a single X chromosome signal as two signals. For this reason, embryo misdiagnosis is extremely unlikely using FISH for sex-determination. There has been one case of misdiagnosis reported to the ESHRE PGD Consortium (Harper et al, 2007), which was associated with social sexing; the pregnancy was terminated. The full aetiology of this misdiagnosis is unknown, but in addition to the accuracy of scoring FISH signals, other potential sources of error include: polymorphism of the probe binding regions, single cells with more than one nucleus or nuclear fragments, contamination with nuclei from maternal cumulus cells associated with the ovum, and incorrect embryo identity at transfer.

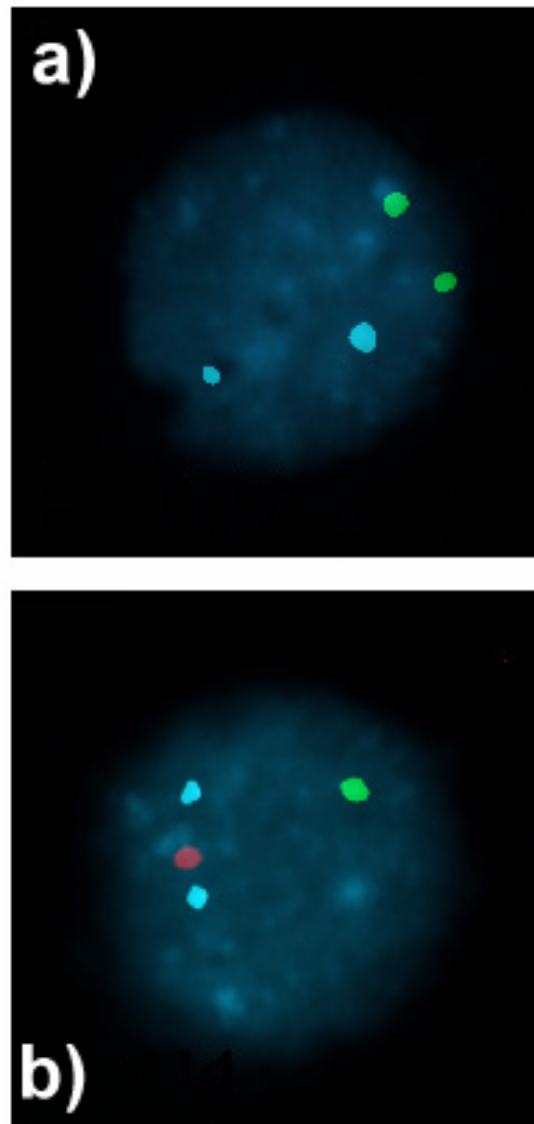


Figure 1: Sexing using FISH. Vysis AneuVysion alphasatellite probes for the centromeres regions of the X chromosome (SpectrumGreen), Y chromosome (SpectrumOrange) and chromosome 18 (SpectrumAqua). a) Normal female (XX,18,18) b) Normal male signal pattern (XY,18,18)

In addition to establishing the sex of the embryo, this test will also identify abnormalities of sex chromosome copy number (for instance a single X chromosome, which is associated with Turner syndrome, and XXY, which is associated with Klinefelter syndrome), as well as embryos with aneuploidy (monosomy or trisomy) for the autosome. The presence of an autosome probe indicates the ploidy of the

embryo, which allows discrimination between a single X chromosome and haploidy (one copy of every chromosome), XXX or XXY and triploidy (three copies of every chromosome) and XXXX or XXYY and tetraploidy (four copies of every chromosome).

An elegant FISH approach to PGD for DMD tests for the deletion of exon 45, the causative mutation in approximately two thirds of DMD cases, by using an exon 45-specific FISH probe, in combination with probes for the X and Y centromeres (Malmgren et al., 2006), making it possible to distinguish between affected and unaffected male embryos and between carrier female and normal female embryos.

#### *PCR - Sexing only*

PCR can be used to amplify a specific repeat on the Y chromosome to sex embryos. However, failure of the Y chromosome sequence to amplify in some cases can and has led to misdiagnosis (Hardy and Handyside, 1992). This approach is not recommended (Thornhill et al., 2005) and is now used infrequently by centres that contribute data to the ESHRE PGD Consortium (Harper et al. 2008).

#### *PCR – Mutation-specific diagnosis*

The disadvantage of the FISH-based sexing approach to X-linked disease is that all male embryos are discarded; on average, half of these discarded embryos will be normal males, and the potential population of embryos available for transfer is diminished. For this reason, PCR methodology has been used to develop direct tests for known mutations associated with a range of X-linked diseases (Harper et al., 2008) (Verlinsky et al., 2002). These protocols generally utilise a nested PCR approach, to include one, two or three microsatellite markers closely linked to the disease gene, in order to monitor contamination and allele drop-out, potential sources of misdiagnosis when using PCR-based testing.

#### *PCR – Preimplantation Genetic Haplotyping (PGH)*

Work-up of specific mutation tests such as those described above can be lengthy and technically challenging, and for many PGD centres, beyond the scope of their funding and staffing constraints. The introduction of PGH (Renwick et al., 2006) has allowed more rapid development of indirect tests of X-linked disease. PGH relies on the identification of the chromosome carrying the familial mutation by haplotype analysis of family members. The presence or absence of this chromosome in embryos can then be established by genotyping the biopsied cells (see Figure 2). This development was made possible by the recent availability of commercial kits allowing extremely effective whole genome amplification, usually by Multiple Displacement Amplification (MDA). MDA is an isothermal technique that uses a  $\phi$  polymerase, which amplifies the single cell DNA approximately  $10^9$ -fold). This amplification then provides sufficient product for the application of multiplex marker panels for genotyping. This approach has the added advantage of effectively “fingerprinting” each embryo, thus obviating the need for washdrop blanks normally required to monitor contamination.

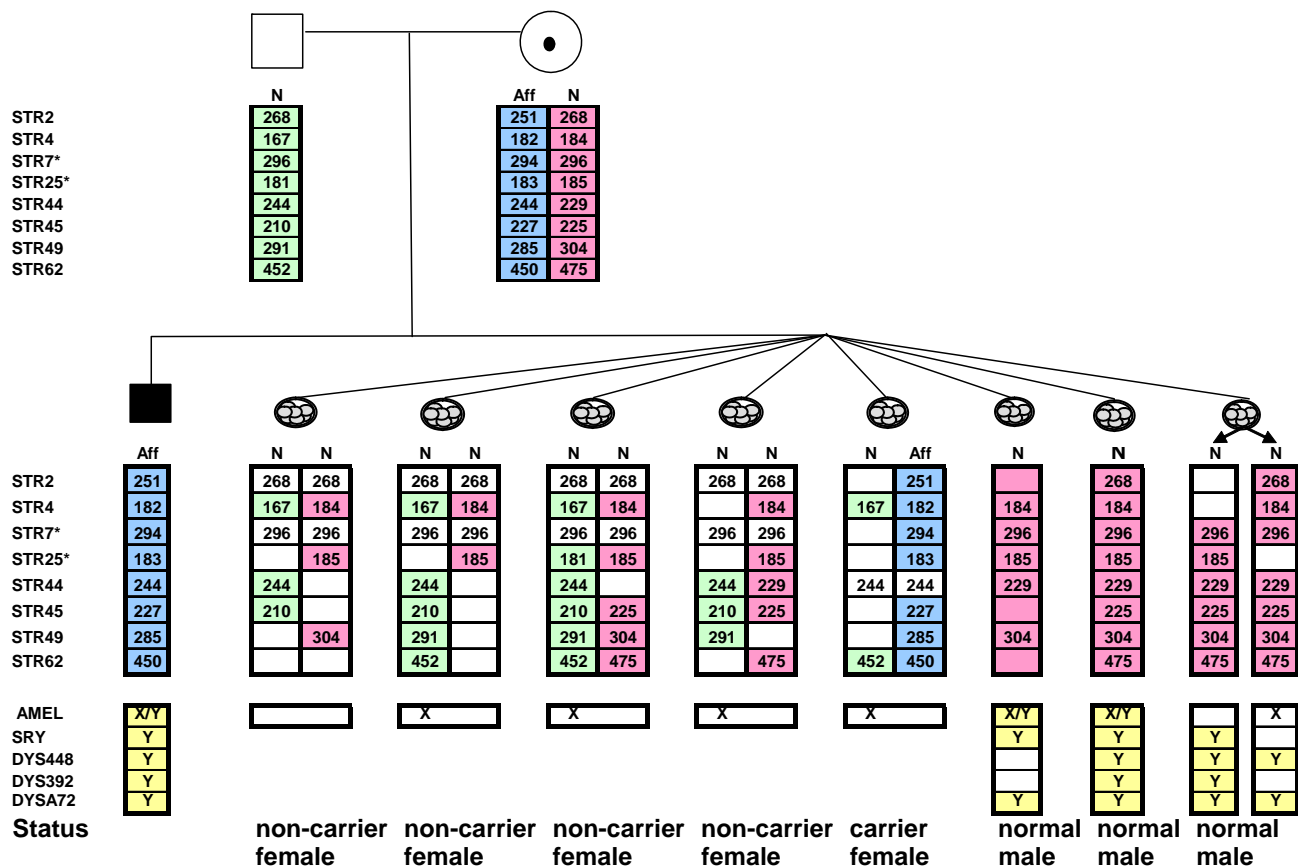


Figure 2: PGH for DMD. Parental haplotypes were inferred from the genotype of the affected child in the family (black square). Green boxes – markers associated with the paternal haplotype; pink boxes – markers associated with the low-risk maternal haplotype; blue boxes – markers associated with the high-risk maternal haplotype. No affected males were present in the embryo cohort in this cycle.

Allele drop-out (ADO) associated with MDA is usually around 20-30%, but may in some cases be as high as 40%, probably due to poor quality starting template, for instance in biopsied cells in which the DNA is degrading; however, the large number of markers that can be tested means that in the vast majority of reported cases, at least one marker is amplified on each side of the disease gene, thus reducing the risk of misdiagnosis to that of a double recombination event (<0.1%). A further advantage of this approach is that the same marker multiplex can be used for all families with the same disease, without the need for the development of family-specific mutation testing. In our experience to date, PGH for DMD results in 56% of biopsied embryos with “transferable” results (normal female, carrier female and normal male) (see Figure 3) compared with 33% when FISH is used for sex determination.

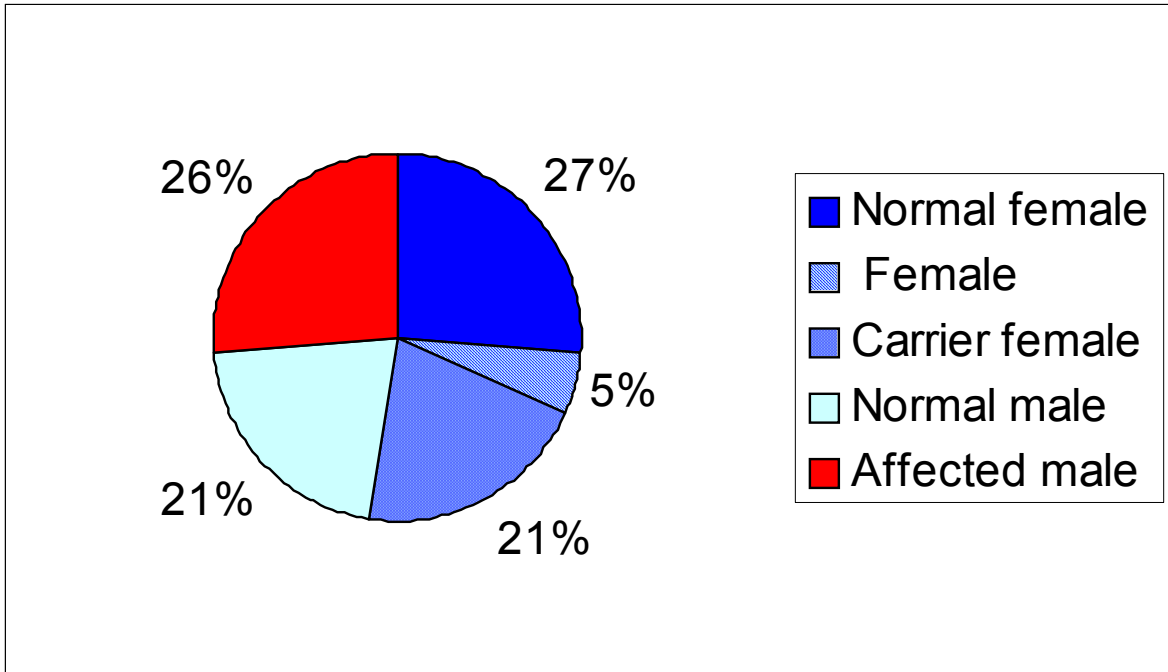


Figure 3: Distribution of embryo categories following PGH cycles for X-linked disease. In cycles where FISH for sex determination is used, the pale blue segment (normal males) would be discarded.

Indirect testing has also been reported using single-cell protocols without prior whole genome amplification. For instance, 6 microsatellite markers were identified in the region of the X chromosome long arm containing the genes for Haemophilia A, Adrenoleukodystrophy, hydrocephalus and IP. These genes are in close proximity to each other, and the authors describe using combinations of these markers, found to be informative for the individual families tested, to identify embryos free of disease (Gigarel et al., 2004). Two cells were biopsied from each embryo, and only those with concordant results were transferred, presumably due to the possibility of ADO or contamination, which may be undetected due to the small number of markers used.

### **Sexing for non-medical reasons (social sexing, gender balancing)**

Many professionals involved in the PGD field take the view that sex selection for a non-medical reason is unacceptable, and few centres reporting to the ESHRE Consortium accept such patients (Harper et al., 2008). Nevertheless, cycles for social sexing continue to be reported to the ESHRE Consortium. The ethical issues associated with this topic are discussed below.

### **Current status of PGD for sex-linked disease and sex determination**

The ESHRE 2004 data collection ([Harper et al., 2008](#)) reports on 816 cycles to oocyte retrieval (OR) of sexing only for X-linked diseases, 225 cycles to OR using a specific diagnosis of an X-linked disease and 412 cycles to OR for social sexing.

The most common diseases tested by centres contributing cycle data to the ESHRE PGD Consortium using a specific diagnosis were DMD, Haemophilia A and Fragile X. The most common X-linked diseases where sexing only was used were DMD, Haemophilia and X-linked mental retardation.

## Ethical Issues

### *Discarding normal male embryos*

PGD for X-linked disease based on identification and transfer of female embryos only is also ethically contentious because of the destruction of potentially normal male embryos. One approach to this dilemma is to sort the sperm prior to the PGD cycle, in order to enrich for X-bearing sperm using flow cytometry (Fugger et al., 1998), and hence to minimise the production of male embryos and increase the number of embryos available for transfer. Flow cytometric separation of X and Y-bearing spermatozoa is still experimental and the subject of an ongoing clinical trial.

### *Unusual PGD requests*

Ethically difficult requests for PGD include those for male selection in X-linked dominant diseases, such as IP, where the mutation is lethal to male embryos, but carrier females are likely to manifest symptoms. By selecting male embryos, only those without the mutation will reach term, but the transfer of embryos that have a 50% chance of abnormality is ethically difficult for PGD staff. Similarly, requests have been made for transfer of male embryos where the male partner carries a mutation of variable penetrance, or an “intermediate” expansion in the fragile X gene, for instance. This strategy ensures that the mutation is not passed to the next generation (all male embryos will inherit their single X chromosome from their mother, and will therefore be free of the mutation), but involves the destruction of female embryos which may carry only a very small risk of phenotypic abnormality. Conversely, selection of female embryos has been requested for families where X-linked mental retardation is suspected from the family history, but the specific aetiology is unknown. In these cases, the condition may not in fact be X-linked, and the association with gender purely coincidental. This possibility means that the decision to accept such families for PGD can be particularly difficult.

### *Allowing choice of sex following PGD for X-linked disease*

Couples embarking on PGD cycles for X-linked disease involving sexing only are clearly aware that any child resulting from a successful cycle will be female. The introduction of direct mutation testing with linked markers, and indirect testing such as PGH, which permit selection and transfer of normal male embryos, has meant that the sex of each embryo is likely to be known at the time of embryo transfer. In the UK, testing to select embryos of a particular sex for social reasons is prohibited by the [HFEA Code of Practice](#). The [Human Fertilisation and Embryology Act \(1990\)](#) empowers an HFEA licence committee to vary or revoke a licence if they consider there has been a failure to observe the code. The code is designed to prevent social sex selection “by the back door”. However, there may be good reasons why, for instance, a male would be preferred to a heterozygote female; while both such embryos would be considered “transferable” by most centres, in some cases there is the possibility that a heterozygote female may show some manifestations of the disease. The legal rights of the couple to have all the information concerning the status of each of their embryos, in order to weigh up the balance of risks for themselves, may outweigh legislation aimed at reducing social sex selection; withholding these rights may be considered unethical as well as illegal. Where a couple has embarked on a PGD cycle because of an X-linked disease in the family, rather than to choose a child of a certain sex, it is usually the case that their over-

riding motivation is to have a healthy baby, regardless of gender. Providing potentially relevant information on the status of their embryos following genetic testing may therefore be considered reasonable and responsible. In any case, only in a very small number of cases is it likely that there will be a choice between embryos of equal morphological quality, and the recommendation of embryos for transfer from the available cohort must continue to be based on morphological considerations. Europe-wide quality control of PGD is currently being formulated, and this is an area that is likely to be covered by any best practice and quality guidelines that emerge.

### *Sexing for non-medical reasons*

The Ethics Committee of the American Society of Reproductive Medicine (ASRM) reported on sex selection and PGD (1999) and recognised that “individuals and couples have wide discretion and liberty in making reproductive choices, even if others object”. The committee identified that sex selection might provide “perceived individual and social goods such as gender balance or distribution in a family with more than one child, parental companionship with a child of one’s own gender, and a preferred gender order among one’s children”; the report also highlighted ethical concerns, which include “the potential for inherent gender discrimination, inappropriate control over nonessential characteristics of children, unnecessary medical burdens and costs for parents, and inappropriate and potentially unfair use of limited medical resources”.

The ASRM report concluded that PGD to prevent the transmission of a serious sex-linked disease is ethically acceptable, and identified four classes of embryo sex identification by PGD for non-medical reasons with varying degrees of ethical risk. The recommendation was made that PGD “should not be encouraged” where the patient learns the sex of an embryo “as part of, or as a by-product of” PGD done for medical reasons; where the patient requests that sex identification is added to the test that is being done for a medical reason, and where PGD is not a necessary part of the treatment for patients undergoing ART. The recommendation was made that PGD “should be discouraged” where ART and PGD are initiated only for the purpose of sex selection.

For some who consider sex selection for non-medical reasons to be unethical, there is no room for flexibility; however, others may see that in some situations their personal viewpoint may conflict with reasonable freedom of choice for the couples involved. It certainly seems excessively harsh to discourage a couple from having PGD for an X-linked condition, for the simple reason that the test will identify the sex of the embryos available for transfer (see previous section). However, nearly all professionals in the field agree with the final recommendation of the ASRM report, viz. that testing should be discouraged (and is in fact regulated against in many countries) where ART and PGD are not required for any other reason than to select the sex of a child.

## **Conclusion**

The introduction and development of new tests for X-linked disease means that the range of these conditions for which PGD is available will continue to widen. However, X-linked disease provides special ethical, counselling, and decision-making challenges beyond those posed by autosomal monogenic defects and chromosome

rearrangements. PGD to determine the sex of embryos for non-medical reasons continues to be illegal or highly controversial in most countries.

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