# Identification of a Gene Responsible for Recurrent Familial Hydatidiform Moles

**Sharlene Murdoch**

**Department of Human Genetics**

**McGill University, Montreal**

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# ABSTRACT

Hydatidiform mole is a form of gestational trophoblastic disease characterized by absence of embryo and hydropic degeneration of the chorionic villi. The majority of complete hydatidiform moles are sporadic and androgenetic; however, a rare subset has been identified which has a biparental genome. These have been found occasionally to recur in the same woman and, in multiple women in the same family suggesting a genetic defect. A maternal locus for biparental familial moles has been mapped to a 1.1Mb region on 19q13.4. Genotyping of a new family with multiple affected sisters has lead to the narrowing of this interval to 0.65Mb, which contains 30 genes. Screening of these genes led to the discovery of mutations in *NALP7* in all affected individuals from four families of different ethnic origin. *NALP7* is a member of a family of cytoplasmic proteins known to play a role in inflammation and the innate immune system.

# RÉSUMÉ

La Môle Hydatidiforme est une forme de tumeur trophoblastique gestationnelle caractérisée par l'absence d'embryon et la dégénération hydropique des villosités. La majorité des môles complètes sont sporadiques et androgénétiques, toutefois il existe un sous-ensemble rare qui sont biparentales. Celle-ci s'est avérées, de temps en temps de se produire à répétition chez la même femme, ou même chez plusieurs femmes de la même famille, suggérant un défaut génétique. Un locus maternel pour les môles familiales biparentales a été localisé à une région de 1.1Mb sur le telomère du bras long du chromosome 19. Le génotypage d'une nouvelle famille avec plusieurs soeurs atteintes à mener au rétrécissement de cet interval à 0.65Mb, qui contient 30 gènes. La recherche de mutations dans ces gènes nous a mené à la découverte des mutations dans *NALP7* chez tous les individus atteints de quatre familles de différente origine ethnique. *NALP7* est un membre d'une famille de protéines cytoplasmiques connues pour jouer une rôle dans l'inflammation et le système immunitaire innée.

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# INTRODUCTION AND LITERATURE REVIEW

"I pulled out a great part of this strange substance, which appeared full of fibres and white bladders full of water, glistening like crystal, and in shape not unlike our white currents."

~ Paul Portal, *17th century obstetrician*[1]

Reproductive wastage and infertility plague one in nine couples in the Western world[2]; this can take on many forms, one of which, is the hydatidiform mole. Many advancements have been made in our understanding of this disorder since the famous seventeenth century obstetrician Paul Portal gave us the first documented description of how to remove moles[1]; however we are still at a loss to explain how and why they form. A better understanding of the cause of many disorders is gained by studying the disease within a familial context. This allows the determination of the underlying genetic defect in families, and points researchers to the cause and consequently the general pathway that leads to the disease. This approach can also be taken for reproductive disorders when a genetic cause is suggested, as has been the case for hydatidiform moles. With birth rates in the Western world dropping and the need to rely on assisted reproduction increasing, the need to understand the causes of infertility and reproductive wastage is paramount.

Hydatidiform mole is a form of gestational trophoblastic disease characterized by the absence of embryonic structures and hydropic swelling of the chorionic villi. This swelling of the villi is what gives moles their distinctive grape-like appearance. At this time there is no adequate way to prevent recurrent moles, although there have been some suggestions including intracytoplasmic sperm injection, prenatal genetic diagnosis and egg donation. These procedures, however, are costly and are not always effective. By

identifying a gene for the familial form of this disorder we can attempt to elucidate the complex pathway that leads to moles and be better able to counsel women afflicted with this ailment. At the present time, it is not definitively known where the defect leading to moles lies, whether it be in the egg or if the problem occurs at implantation. Should the latter prove true, then the use of donor eggs is not a viable treatment option.

This thesis will begin with a review of the relevant literature and an overview of the constantly evolving concepts of what causes molar pregnancies. I will then go on to describe the mapping of a familial locus for this disorder, review the candidate genes located therein and their screening for mutations. Following this is a manuscript that we submitted for publication describing the identification of *NALP7* as the causative gene for familial biparental hydatidiform moles.

## *Overview of Implantation and Placentation*

As the primary tissue affected in molar pregnancies is the chorionic villi, a major component of the placenta, it is important to understand the normal form and function of this specialized tissue. The human placenta is a highly evolved and complex structure, whose main normal role is to facilitate the exchange of nutrients and gasses between mother and foetus[3]. However, it also serves other vital functions; for example, the placenta is the site where the maternal immune system comes into contact with foetal antigens and is the potential site of immunological rejection of the foetus. In addition, the placenta acts as a barrier protecting the foetus from foreign substances. Thus, proper placental development is essential for foetal viability. In this context it is not hard to see that abnormal placental development, like that seen in hydatidiform moles, could lead to very early foetal death or even absence of foetal formation.

## Implantation

The first step of placentation is embryonic implantation, a process that requires interaction between the blastocyst and the maternal reproductive tract. The process of mammalian implantation has mainly been studied in mice, but the same general process can be extrapolated to humans. Once the embryo reaches the site of implantation it lodges in the endometrium and hatches from the zona pellucida, which allows direct contact between the blastocyst and the luminal epithelium. The blastocyst then triggers apoptosis of the epithelial cells[4]. Apoptosis is programmed cell death in which the dying cell is broken down and carefully cleared away by phagocytic cells so as not to release antigens into the blood, which would trigger an inflammatory reaction. The trophoblast cells then cross the basement membrane to invade the decidua. Apoptosis is an important part of implantation, as the embryo must break down the endometrial cells to invade the decidua without triggering an inflammatory response from the maternal immune system, which would, in turn, attack the blastocyst. There is, however, a narrow window during which the uterus is receptive to implantation; it is therefore essential that this window coincide with the readiness of the blastocyst to implant. To this end the uterus secretes growth factors to speed up or slow down blastocyst development. We can see that successful implantation requires the strictly regulated interaction of maternal and blastocyst factors which must act in concert. It follows from this that disregulation at any step along this path may lead to implantation failure or abnormal trophoblast growth. After implantation, apoptosis plays a further role in maintaining the balance of trophoblast proliferation during pregnancy[5]. Deregulation of the apoptotic process can lead to pregnancy complications. Increased apoptosis has been observed in hydatidiform

molar pregnancies[6-8] as well as pre-eclampsia[5]. Thus we can see that extrinsic signals are essential for implantation and proper trophoblast differentiation.

## Implantation and Inflammation

There are clear similarities between the processes of inflammation and embryonic implantation. A key event in the inflammatory process is the migration of leukocytes across the endothelium[9], which has analogies to implantation. Both implantation and the transendothelial migration of leukocytes are tripartite processes comprised of apposition, adhesion and invasion. In apposition, both the leukocyte and blastocyst are acted upon by cytokines and chemokines to guide them to their proper location: the implantation site for the blastocyst and the inflammation site for the leukocyte. In adhesion, ligand-receptor interactions are responsible for the anchoring of the blastocyst to the implantation site and the leukocyte to the site of inflammation. Lastly, just as the leukocyte must then squeeze through the tight junctions to transverse the endothelium in a process known as diapedesis, so too must the blastocyst transverse the endometrium during trophoblast invasion. Trophoblast invasion differs from leukocyte migration, in that the blastocyst triggers apoptosis of the endothelium[10]. The inflammatory response is the way by which our innate immune system protects us from invading pathogens such as viruses or bacteria. Interestingly one of the major ways by which bacteria trigger an inflammation response, the shedding of LPS, is also a known cause of pregnancy loss. Lipopolysaccharide, or LPS, is a toxin found in the cell walls of gram-negative bacteria[11]. This toxin stimulates the endometrial macrophages to release cytokines including the interleukins 1$\alpha$ and 1$\beta$. Through this cytokine release LPS can adversely affect

pregnancy at many different levels including blastocyst growth, trophoblast development, implantation, and later in pregnancy cause spontaneous abortions[11].

### Placental Morphology

The placenta and extra-embryonic membranes are derived from a lineage of blastocyst cells, the trophectoderm, which is distinct from the cells that will form the embryo. These cells differentiate into the trophoblast[4]. Trophoblast cells are classified into two groups based on their location in the placenta. Villous trophoblast cells form the chorionic villi and are formed by three cell types: cytotrophoblasts, intermediate trophoblasts and syncytiotrophoblasts. Extravillous trophoblast cells infiltrate the decidua and are composed mainly of intermediate trophoblasts[12]. Abnormal growth of trophoblast cells leads to a condition known as gestational trophoblastic disease (GTD). GTD is a broad term used to describe a range of conditions including choriocarcinoma, placental site trophoblastic tumour, hydatidiform mole and invasive mole[13]. Of these, hydatidiform moles are the most common.

## *Hydatidiform Moles*

### Presentation and Pathology

Hydatidiform moles present in the first trimester mainly with vaginal bleeding, large uterine size for gestational age, and highly elevated serum levels of human chorionic gonadotrophin (hCG)[14]. Moles occur in two forms, partial (PHM) and complete (CHM), with the distinction between the two being made based on karyotype and histopathology.

Partial moles are triploid, normally having two copies of the paternal genome. Microscopic analysis of partial moles reveals two types of chorionic villi: normal and hydropic[15]. Partial moles also tend to be associated with some foetal tissue. Complete moles are diploid and usually androgenic. All of the villi in complete moles show hydropic changes and no foetal tissue is present.

Mechanisms have been proposed to explain the origin of partial and complete moles. It has been suggested that most partial moles arise by dispermy: the fertilisation of a normal ovum by two sperm, and that complete moles occur via the fertilization of an empty ovum either by two sperm, or by a single sperm which then duplicates[13]. The existence of a so-called "empty ovum" has yet to be demonstrated, but it is thought that if the egg is not empty, at the very least its nucleus is inactive, rendering it, for all intents and purposes, empty. Another class of complete mole has been identified, which has a biparental genome but it is phenotypically indistinguishable from its androgenetic counterpart. Complete moles pose a higher risk of developing into persistent trophoblastic disease than do partial moles[16].

## Diagnosis and Treatment

Diagnosis of moles is made based on ultrasound, which reveals the presence of echogenic cysts and no foetus. Moles are removed by suction curettage, and the patient's hCG level is measured weekly until it falls down to a non-pregnant level. In some cases, the hCG remains high after the evacuation of the mole and indicates a persistent trophoblastic disease (PTD)[14]. Hydatidiform mole is also a tumour, which has the ability to become invasive and may metastasize, forming a choriocarcinoma. Molar pregnancies carry with

them a 2000 to 4000 times greater risk of developing choriocarcinoma than do normal pregnancies[13].

There is, at this time, no completely effective method of preventing the occurrence of further moles for women with recurrent molar pregnancies. Logically, there are common assisted reproductive methods which could circumvent the formation of a mole, such as intracytoplasmic sperm injection (ICSI), which will eliminate fertilisation of the egg by two sperms, avoiding a triploid partial mole, and preimplantation genetic diagnosis (PGD) to select for 46, XY embryos preventing androgenetic complete moles[17,18]. These treatments, however, have their shortcomings, as ICSI does not prevent partial moles that may arise from duplication of the paternal genome, and PGD does not distinguish diploid androgenetic embryos that have arisen due to eggs that have been fertilised by two sperms carrying different sex chromosomes. Indeed, cases of moles, both partial and complete, following ICSI have been reported[19-21]. Neither of these options can prevent the occurrence of biparental complete moles since these arise from seemingly normal conceptions. It should be noted that the use of ICSI or PGD, while theoretically could prevent moles are not routinely prescribed as a course of treatment for women with recurrent moles. The cases of moles following ICSI were random events and they did not occur in a recurrent or familial context, however, they serve to demonstrate the limitations of this procedure. At this time only two cases have been reported in the literature of in-vitro fertilisation (IVF) being used in an attempt to treat women with recurrent moles[22,23]. In one case no embryos were produced that were suitable for transfer; however, only preliminary tests were done as the patient chose to forgo further treatment[22]. In the other case a woman who had had a history of moles with

a previous partner sought IVF treatment to conceive with a new man. She underwent two rounds of IVF and each time an unusually high number of the fertilised oocytes were triploid; however, some embryos did form normally and were transferred, although none resulted in a pregnancy, molar or otherwise[23]. A third option suggested has been the use of donor eggs[23-25]. This is based on the assumption that the defect leading to moles is confined to the eggs of the affected women. This option, however, is not available to all women and, in some parts of the world, it is even illegal[25]. Regardless, there has been no report of its use or efficacy in avoiding recurrent moles.

## Incidence and Epidemiology

Hydatidiform moles can occur in a sporadic or familial context with sporadic moles being more common. In North America, moles occur with an incidence of 1 in 1500 pregnancies and most of these are sporadic cases. The incidence varies, however, in other populations across the globe. Rates of molar pregnancies are highest in South East Asia, reaching approximately 1 in 83 pregnancies[26]. Incidence is also higher in the Middle and Far East as well as Latin America. Maternal age has been identified as a risk factor for sporadic moles with women at extremes of reproductive age (teenagers and women over 40 years of age) having the highest risk[26]. This is true for both complete and partial moles, but the risk is higher for complete moles[16]. The risk of recurrence of a molar pregnancy is 1% if a woman has had one mole, but jumps to 25% if a woman has had two or more[15]. No other link has been shown between risk of hydatidiform moles and any other factors, such as diet or blood type. However, a statistically significant link has been found with spontaneous abortions: risk of gestational trophoblastic disease increases with an increasing number of spontaneous abortions and this trend is consistent

across all populations and regardless of maternal age[27]. Conversely, the risk of gestational trophoblastic disease tends to decrease with an increase in the number of live children a woman has given birth to[27].

### *Moles and Genomic Imprinting*

Genomic imprinting is a phenomenon in which only one parental allele of a gene is transcribed or expressed, and is one of the most commonly studied epigenetic modifications in mammals. This phenomenon is regulated by several mechanisms of which the most extensively studied is DNA methylation. Imprinted genes are usually found in clusters, and are regulated by upstream elements known as differentially methylated regions (DMRs). These regions are controlled by imprinting control centres, analogous to what has been observed for X chromosome inactivation in female mammals[28]. When a gene is paternally expressed it is said to be maternally imprinted, while if it is maternally expressed it is paternally imprinted. The inactive copy of the gene tends to be hypermethylated on its DMR and thus silenced, while the active copy is not methylated. In the germline imprints are erased and new imprints are then re-established based on the sex of the gametes[28]. These new imprints are laid down early in development, either during gametogenesis (primary imprints) or at the blastocyst stage (secondary imprints)[29]. In somatic cells the pattern of imprinting is then maintained throughout each round of DNA replication[28]. That the maternal and paternal genomes do not contribute equally to embryonic development was shown by early pronuclear transplant experiments in mice to create androgenetic and gynogenetic embryos. These experiments showed that androgenetic embryos had excessive proliferation of the trophoblast and embryonic death, as is seen in human androgenetic moles. Gynogenetic

embryos, on the other hand, were structurally normal, though none developed to term, while extraembryonic development was abnormal[13,30,31]. Proper genomic imprinting is thought to be essential for proper embryonic development, as most imprinted genes characterized thus far have an effect on development. Much of what is known about the role of genomic imprinting comes from work being done in mice; however, it has also been shown that deregulation of imprinted genes can lead to human diseases; such as Prader-Willi and Angelman syndromes[32].

Since complete moles are identical, whether they are biparental or androgenetic, it is thought that they too may be caused by an error in genomic imprinting. Several studies have been undertaken to evaluate the extent of imprinting defects in moles. In 2002, Judson et al. looked at methylation of a series of imprinted genes in a single biparental mole[33]. They examined methylation of seven genes with maternally methylated DMRs and two genes whose DMRs are paternally methylated. Seven of the DMRs, showed abnormal methylation, with both copies showing a paternal methylation pattern. Two DMRs displayed normal methylation, and one was inconclusive due to conflicting results on the controls. From these results, the authors concluded that moles are caused by a global defect in imprinting in the maternal germline, however, this remains to be conclusively proven. We note that the mole evaluated in this study is from a family in which the defect is not linked to 19q13.4, a candidate locus identified for familial hydatidiform mole.

Another study looked at the expression of $p57^{KIP2}$ in a series of biparental moles from women with a defect linked to chromosome 19. $p57^{KIP2}$ is a protein expressed in the placenta and encoded by a maternally expressed gene[34]. They found the protein to be

underexpressed in biparental moles showing the same expression pattern as androgenetic complete moles, from which they inferred that the gene encoding the protein was abnormally methylated[34].

Similarly, our group studied the methylation of four imprinted genes: *PEG3* and *SNRPN* (both paternally expressed) and *NESP55* and *H19* (maternally expressed) in four biparental complete moles. Two of these were from two sisters with defects in 19q13.4 and two were sporadic, from unrelated patients. We found that the paternally expressed genes had a low level of methylation, while the maternally expressed genes showed increased methylation as compared to normal chorionic villi of matching gestational age. Thus, the biparental moles, in general, showed a paternal methylation pattern, similar to the androgenetic moles[29]. Therefore, all of the studies looking at methylation patterns in biparental complete moles seem to have shown that these conceptions follow a paternal epigenetic pattern, which seemed to support the idea that genomic imprinting errors play a role in the formation of moles. Furthermore, in addition to studying the pattern of methylation, we were able for one gene, *H19*, to use informative SNPs to trace the transmission of the abnormally methylated allele from the grandparents to the moles of the affected sisters. One of the moles showed abnormal methylation on the allele inherited from its maternal grandmother. This allele was unmethylated in the mother, as expected, but gained abnormal imprinting marks in the mole, indicating that the defect was not in the failure to erase parental imprints in the primordial germ cells of the patients[29]. To follow up on our initial work, our group looked at the same imprinted genes, *SNRPN, PEG3, NESP55* and *H19* in the sisters affected with familial moles to see if they have abnormal methylation in their somatic tissues. Our results show normal

levels of methylation at these four genes in blood DNA from these women. As in the first paper we were able to trace the inheritance of the abnormally methylated allele in the moles, this time on *NESP55*, in a second mole from this family[35]. Once more, we saw the gain of abnormal methylation marks in the mole on the allele inherited from its maternal grandmother. Again this allele is unmethylated in the mother as is normally expected. This result suggests that these women do not have general errors in their methylation pathway, rather the abnormal imprinting seen in the molar tissues occurred either in the patients' germline or postzygotically in the moles themselves[35]. At this point it is unclear whether the abnormal imprinting observed in molar tissues is the cause or a consequence of the molar phenotype. Moles are, in essence, tumours, and the presence of abnormal imprinting in tumour cells has been well established. Moreover the pattern of abnormal gene methylation tends to be specific to the tumour type, whether it be hereditary or sporadic. It is thought that the abnormal imprinting may increase the growth potential and transforming ability of the cells, but that it is not necessarily the cause of its oncogenesis[36]. Considering the abnormal methylation in this context allows us to speculate about the implications of the methylation data on moles. The theory of imprinting defects as the cause of molar pregnancy began with the phenotypic link between androgenetic and biparental moles. The subsequent discovery of abnormal methylation at imprinted loci gave credence to this theory. There may be, however, another way to look at it. Perhaps these results –the similar patterns seen by the different groups- point, not to the source of the phenotype, but rather to a molar (tumour) specific methylation pattern. Perhaps this molar specific deregulation of imprinted genes may reflect the end product of a selection process in which the only viable changes happen to

be those, which culminate in the molar phenotype; but does not signify the root cause of its aetiology.

### *Molar Pregnancies and Immunology*

Immunology has long been thought to play a role in pregnancy. A foetus is a semi-allograft within the mother's body, yet it is not rejected by her immune system, as an allogenic transplant would be. Thus pregnancy presents an "immunological paradox" that reproductive immunologists have long sought to decipher[37]. Since the placenta mediates the interactions between mother and foetus, it is thought that this organ plays a central role in the immunological acceptance of the foetus by the mother[37]. Long before the currently held theory that moles are caused by a maternal imprinting defect, it was believed that immunological incompatibility lay at the root of the molar phenotype. An early theory about the formation of hydatidiform moles dates back to 1940 when it was proposed that spontaneous abortions and hydatidiform moles go hand in hand. It was hypothesised that early in gestation the embryo would die and be lost as a spontaneous abortion but the trophoblast would remain and continue to proliferate as a hydatidiform mole[38]. It was not known why in some cases a simple spontaneous abortion would occur while in others it would progress to a mole, but in 1971 Takeuchi proposed that immunological selection could explain this[38]. He later proposed a mechanism by which this could occur. In his 1980 study, he looked at a series of immunological factors in spontaneous abortions and hydatidiform moles as compared to normal pregnancies and he found many differences between the two. Amongst these were that blocking factors were absent in spontaneous abortions but in hydatidiform moles, as in normal pregnancies, they increased with gestational age. He concluded that a "blocking

antibody" was responsible for the proper development of the trophoblast and that this blocking antibody was bound to the trophoblast and protected it against destruction by the maternal immune system. He suggested that "survival or cytotoxic destruction of trophoblast is a strictly immune dependent phenomenon"[38]. He further speculated that it is changes in the amount of blocking antibody that are responsible for the different phenotypes seen in spontaneous abortions versus hydatidiform moles.

Labarrere and Althabe later picked up on Takeuchi's theory, explaining that decrease in blocking antibody would damage the trophoblast and lead to spontaneous abortion but that after the embryo had died there would be a continued production of blocking antibody that would lead to the development of a hydatidiform mole[39]. They also went on to suggest that primary chronic abortion, pre-eclampsia, idiopathic intrauterine growth retardation, hydatidiform mole and choriocarcinoma share a common immunological cause. They theorized that the difference in phenotype between these conditions is due to the difference in the degree of maternal response to the blocking antibody[39].

### *Molar Pregnancies and Pre-eclampsia*

Hydatidiform moles share many features in common with another gestational abnormality: pre-eclampsia. Pre-eclampsia is more common and has been more extensively studied and looking to the root of some of its clinical manifestations can perhaps shed some light on what may be occurring in the pathology of moles. Features the two conditions have in common include increased oxidative stress[40] and increased apoptosis[5,6]. In addition, a 1988 literature review found fifty-seven reported cases of the co-occurrence of pre-eclampsia and molar pregnancies over 120 years[41]. It is estimated

that the risk of developing pre-eclampsia with a molar pregnancy is 25%[42]. In pre-eclampsia it had been hypothesised that the observed increase in apoptosis is a consequence of increased proliferation of the trophoblast, which must be balanced. It is thought that as the tissue tries to keep pace with the excess proliferation, the apoptotic process becomes overwhelmed and what results is a truncated version of apoptosis, which is termed aponecrosis, in which cells initiate the apoptotic process but then switch to necrosis. Necrosis is the opposite of apoptosis. Instead of the proteins and enzymes of the dying cell being carefully packaged and cleared away before they can harm the surrounding tissue, the cell is broken open and its contents freely spilled out. This would lead to the release of foetal proteins into the maternal blood system, cause damage to surrounding cells in the maternal endometrium, and initiate an inflammatory response. These are all characteristics of pre-eclampsia[5].

### *Genetics of Moles*

Early studies into the genetics of hydatidiform moles looked at their chromosome content. Using the technology available at the time, several studies had shown that the majority of moles possessed a Barr body: the inactive X chromosome commonly seen in female cells. These studies, however, had only looked at a small sample of moles[43]. In 1968 Baggish et al., having undertaken a larger scale study which looked at ninety hydatidiform moles in an attempt to confirm the earlier reports, also found the majority of moles to be female[43]. Kajii and Ohama later showed that moles are androgenetic in nature, meaning that their entire chromosome content is paternally derived. They karyotyped twenty moles and found them to be 46,XX. They then looked at Q and R band polymorphisms in seven moles and the parents. With these markers they determined

the moles were homozygous and it was concluded that all of the chromosomes in the moles were of paternal origin[44]. As previously mentioned, there are two theories as to how androgenetic moles can arise: either by dispermic fertilisation of an "empty" egg (an ovum which contains no nucleus or a nucleus which is inactive) or by fertilisation by a single sperm which then duplicates. There exists, however, a third alternative, that of fertilisation by a single sperm that is diploid due to non-disjunction during meiosis. Dispermic fertilisation could not explain the moles seen here for with dispermy one would expect to see some heterozygous markers, whereas all moles studied were homozygous at all markers. The other two possibilities could not be distinguished in this study; however, it was noted that either possibility would result in either a 46,XX or 46,YY conception[44]. Since 46,YY conceptions are non viable, only 46,XX androgenotes would survive to form moles, thus explaining why the majority of complete moles have been shown to possess Barr Bodies[43,44]. These results, showing the androgenetic origin of moles, have been confirmed using a variety of methods including the typing of highly polymorphic HLA markers[45]. However, while this has held true for most cases, it was noted that occasionally moles were found to have a biparental genomic contribution. In 1980, Jacobs et al. studied twenty-four complete moles and found that one of them, while it did have a 46,XX karyotype, was consistent with a normal conception, meaning that it was diploid and biparental. Another mole in the study was 46,XY; however, they could not determine if it was the product of a normal conception or if it arose via the fertilisation of a empty egg by two different sperm[46]. Subsequently, several cases of biparental moles have been reported[17,47,48]

## *Sporadic versus Familial Moles*

While the majority of cases of hydatidiform moles are sporadic, occasionally they have been found to recur in the same woman and, in rare instances, in several women from the same family. The first familial cases of recurrent moles were reported by Ambani et al. in 1980[49]. While cases of familial moles are still rare, since the initial report several more cases of recurrent familial moles have been identified with many of these families displaying consanguinity between the parents of the affected women. Analysis of all recurrent moles, sporadic as well has familial, has found them to be biparental[24]. That moles have been found to recur in families has suggested an underlying genetic cause. It has been suggested that the, as yet, undetermined genetic defect segregates in an autosomal recessive manner, owing, in part, to the consanguinity (the parents of the affected women are often first cousins) observed in these families[47].

### Note on familial and sporadic designations

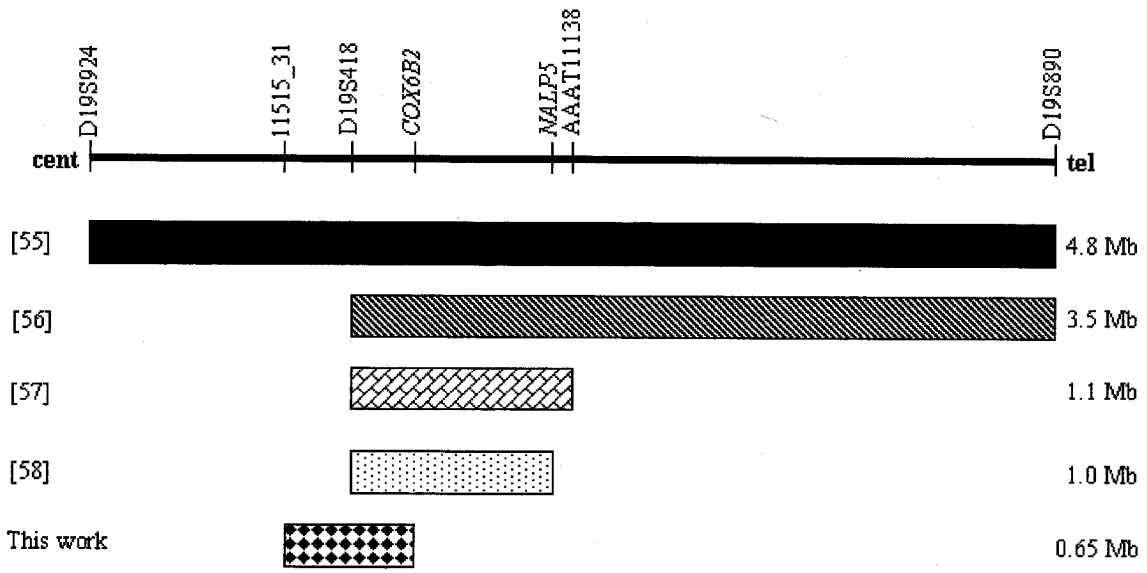
The number of reported families affected with recurrent moles is small. However, it should be noted that women with recurrent moles who are deemed "sporadic" are affected with the same pathology. In order for a case to been deemed "familial" we must see multiple women in the family affected with recurrent moles, but in some cases a woman may have multiple moles who has no female siblings, or whose female siblings have yet to try to conceive, thus their status with regard to molar pregnancy is unknown. In these cases the women are, by default, deemed sporadic. Nevertheless the recurrence of biparental moles in these patients puts them in the same category as the familial cases. As such, screening these women may also help to identify the causative gene.

## *Mapping of a Maternal Locus for Recurrent Hydatidiform Moles*

While, as previously mentioned, the majority of cases of molar pregnancies are sporadic, they have, in rare incidences, been shown to recur in families. The first such report came in 1980 when Ambani et al. noted three different families with affected sisters[49]. Other groups over the next few years reported two more familial cases[50,51]. The recurrence of this disorder in five families pointed definitively to an underlying genetic cause and, consequently opened up a new area of research. A consanguineous Lebanese family was reported in which two sisters and a cousin were affected with multiple moles (family MoLb1). The two sisters, both married to their first cousins, were reported to have had multiple moles and spontaneous abortions, though the younger of the two had succeeded in giving birth to a normal healthy daughter[47,52]. Another member of the same family had been reported by another group; this woman had experienced four consecutive moles, and a fifth pregnancy which resulted in a healthy boy[48,53]. This patient is a cousin of the two Lebanese sisters and is also married to her first cousin. Analysis of the moles from these three women revealed them to be of biparental origin[47,53]. In another familial case, a German family was reported with three affected and three unaffected sisters (family MoGe2); however, no consanguinity was reported in this family[54,55]. In 1999, our group performed a genome wide scan on these two families to map a locus for familial hydatidiform moles. Based on homozygosity, a candidate locus was mapped to a 15.2 cM region of chromosome 19q13.3-13.4 between markers D19S924 and D19S890[55]. Subsequently, genotyping of additional families helped to refine this region (figure 1). In 2000 Sensi et al. reported an Italian family with two affected sisters, who had each experienced multiple moles and spontaneous abortions, but no normal pregnancies[56].

This family was found to be linked to the region and served to narrow down the proximal border of the candidate region[56]. The limit was redefined based on heterozygosity at a single marker in the candidate region in both sisters. This discovery resulted in an exclusion of 2.8 cM from the proximal border of the candidate locus[56]. Fisher et al. reported a case of two sisters from a consanguineous marriage who had experienced biparental complete moles (family CX01). Although this family was linked to the region, it did not serve to narrow it down [34]. The same authors later described another family (CX02) of European origin with two affected sisters; this family was also linked and allowed them to narrow down the locus at its distal end, placing the size of the candidate locus at 1.1 Mb[57]. Most recently a new family with linkage to chromosome 19 was reported[58]. This case is a consanguineous family of Egyptian origin with three affected members: two sisters and their cousin. The authors also report a possible familial case of two affected cousins of Egyptian origin, and three sporadic cases, all of which are linked to 19q13.4[58]. One of these women had a heterozygous polymorphism within the *NALP5* gene which allowed for a slight narrowing of the locus at its distal border, giving a candidate region of 1.0 Mb[58]. We recently analysed a family from Pakistan (MoPa61). This is a consanguineous family with three affected sisters. The oldest sister had fourteen moles and another sister had a total of nine moles with two different partners[59]. Genotyping of this family allowed us to further narrow the distal border of the locus to within the gene *COX6B2*.

**FIGURE 1: REFINING THE CANDIDATE REGION**




**Narrowing down of the familial hydatidiform mole locus on 19q13.4.** Markers and genes are ordered from the centromere on the left to the telomere on the right. The markers that have served to designate the boundaries of the region in each of the papers are listed in their relative order along chromosome 19. The overall size of the candidate region defined by each paper is given in megabases on the right.

## *Families Not Linked To 19q13.4*

As is the case with many genetic disorders, not all families with recurrent moles have a defect in 19q13.4. This is evidenced by the reports of familial cases that do not show linkage to the region; thus far, two such families have been reported. In 2002, Judson et al. reported a consanguineous family from Pakistan in which two cousins and their aunt each had multiple moles and no normal pregnancies. This family was shown not to display homozygosity for the candidate region[33]. Our group recently reported a family from Iran in which four sisters and a cousin were affected with molar pregnancies (family MoIr56). The eldest of the four sisters had three normal pregnancies and two complete moles; the second sister had one partial mole and two spontaneous abortions; the third

sister had one spontaneous abortion, two complete moles, and a normal pregnancy; while the fourth sister and the cousin each had one complete mole[60,61]. This family was likewise shown not to display linkage to 19q13.4 and, in addition to indicating the existence of a second causative locus for this disorder, this family displays a wide clinical variability in the phenotype of the conceptuses. MoIr56 is currently being used to map the second locus for familial hydatidiform moles.

### *Identifying Candidate Genes*

As theories regarding the aetiology of moles evolve, so too must the concept of what type of gene would cause this disease. The hydatidiform mole is an enigmatic disease whose pathological findings give us little to point to an overt cause for its phenotype. As such, it is difficult to determine what would make the best candidate gene. Advocates of genomic imprinting would favour a gene involved in the establishment of methylation marks and would suggest oocyte specific transcription factors or DNA binding proteins that would play a role in the maintenance of methylation marks or the conformation of chromatin. One group has indeed screened three DNA methyltransferase genes: *DNMT3L*, *DNMT3B*, and *DMNT2* (though they are not located on 19q13.4) in a case of familial recurrent moles but found no mutations[62]. Immunologists and pathologists, on the other hand, would suggest immune related genes or genes involved in trophoblast differentiation. The problem inherent to such an intricate process is that a valid argument could be made for virtually any gene in the candidate region. For this reason, it was very difficult to prioritise gene screening and we had to screen all genes in the candidate region. The process was further complicated by the fact that chromosome 19 is the most

densely gene packed chromosome in the human genome[63], making gene screening a very lengthy process.

## *Gene Screening*

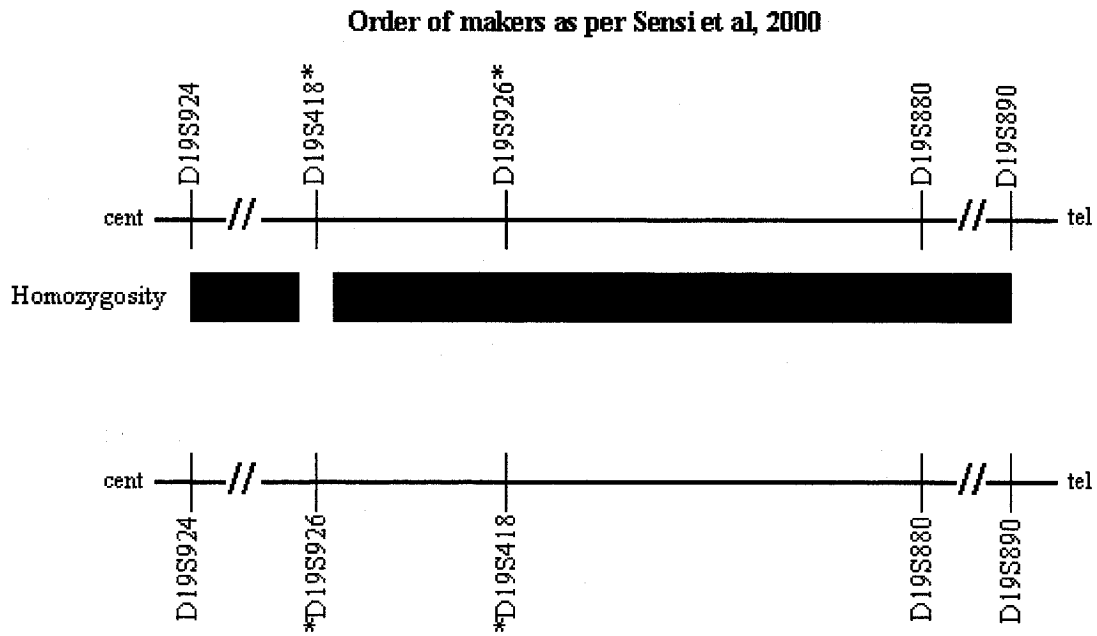
The 1.1 Mb candidate region contained more than sixty genes. At the time I embarked on this project ten of these genes remained to be screened, the others having already been excluded. I completed the screening of these genes, including all of their predicted splice variants, in two patients: one each from MoLb1 and MoGe2, but found no mutations. It was at this time that our laboratory received DNA from family MoPa61 and evaluated its linkage to 19q13.4. As previously mentioned, we found it to be linked and were able to narrow down the size of the candidate region at the distal border. The region now contained fourteen genes, all of which had been screened and excluded. For this reason we re-evaluated the mapping data, taking a closer look at the positioning of the proximal border.

### Redefining the proximal border

The two sisters affected with recurrent moles studied by Sensi et al. were homozygous for every marker in the candidate region defined by Moglabey, save one: marker D19S418[56]. The genetic order of the markers in this region was centromere-...-D19S418-D19S926-D19S880-...-telomere; lack of homozygosity for D19S418 therefore defined it as the new proximal border for the familial hydatidiform mole locus. However, it should be noted that at that time the physical map of chromosome 19 was not complete and it has since been determined that the actual order of the markers used to define this border are not what they were thought to be at the time. It has since been determined that

D19S418 is actually located between D19S926 and D19S880 (figure 2). Owing to the confusion over the placement of the markers, and that for all families we ourselves evaluated homozygosity extended beyond D19S418, we base our proximal border on that defined by MoLb1[55].

**FIGURE 2: POSITIONING OF THE PROXIMAL BORDER**




**Defining of the candidate region by Sensi et al., 2000.** On top is the genetic order of the markers studied by the authors. Markers were tested for the whole 4.8 Mb candidate region as defined by Moglabey et al., stretching from D19S294 to D19S890. The black bar indicates the extent of homozygosity in affected members of the family studied. The affected sisters were homozygous for all markers except D19S418. On the bottom is the physical order of the same markers according to the May 2004 assembly on the UCSC genome browser. http://genome.ucsc.edu/cgi-bin/hgGateway. The disputed markers are identified with an asterisk (*).

To more precisely define this border in MoLb1, we derived new markers for fine mapping and have identified marker 11515_31 (at 27 467 Mb along contig NT_011109) as the new proximal border of the candidate region. The new distal border is at exon 3 of

the gene COX6B2 (at 28 133 Mb along contig NT_01109) as defined by MoPa61. Therefore, the newly defined candidate region is 0.65 Mb and contains thirty-five genes. Two of these, *TNNT1* and *TNNI3*, are known to be responsible for other diseases (Amish nemaline myopathy OMIM 605355, and familial hypertrophic cardiomyopathy OMIM 192600, respectively) and were thus not considered candidates. The genes in the newly extended region are immune or immune related genes: the *KIR* gene cluster (a family of receptors for MHC class I ligands), *NCR1*, *FCAR* and *NALP7* (also known as *PYPAF3* or *NOD12*). As previously mentioned, there has been a long suspected link between immunology and the development of moles; therefore, in addition to these genes now being in the candidate region, they also made interesting and logical candidates. Of particular interest to us were the KIRs, which have long been studied for a possible link to reproduction. Owing to the high degree of homology between the KIR genes, designing specific primers and obtaining clean sequences for each of the fourteen expressed genes was an arduous and time consuming task. Before embarking upon it, we screened the other immune related genes in the region that were likewise good candidates: *NCR1* and *FCAR*.

### *NCR1*

This gene codes for the Natural Cytotoxicity Triggering Receptor 1, which belongs to a family of receptors found on the surface of Natural Killer cells. These receptors are responsible for triggering the killing of HLA-class 1 negative cells, such as tumour cells. *NCR1* was the first of this family to be identified and blocking its activity was shown to inhibit the cytolysis of various tumour cells[64]. While these receptors have also been shown to trigger the killing of virus infected cells[65], it is their ability to recognise tumour

cells that would have made them interesting candidates to be involved in the aetiology of moles. A defect in NCR1 could explain why the molar tissue, which is essentially tumour tissue, is not recognised and killed. However, this gene was screened and no mutations were found.

### *FCAR*

This gene encodes another immune receptor: the Fc-alpha Receptor. FCAR is involved in the inflammatory pathway and is expressed on the surface of neutrophils and macrophages[66]. This receptor binds IgA, a common immunoglobulin which protects mucosal surfaces from bacterial invasion[67], and can trigger cytolysis or the release of inflammatory cytokines[66]. Polymorphisms in this gene have been associated with IgA nephropathy, a condition characterized by deposits of IgA complexes in the glomeruli that may lead to kidney failure[66]. Given the similarities previously discussed between implantation and inflammation, the role of *FCAR* in inflammation may have made this gene an interesting candidate; however, its implication in another disorder made it unlikely to play a role in molar pregnancy. Despite this doubt, this gene was screened, but again, no mutations were found.

### *Killer Immunoglobulin-like Receptors*

Killer Immunoglobulin-like Receptors (KIRs) are also a family of membrane bound receptors which are found on the surface of Natural Killer (NK) cells and some T cells and bind HLA class I molecules. They come in two forms: activating or inhibitory. The inhibitory receptors have been more extensively characterized than their activating counterparts. These inhibitory receptors block the activation and function of NK cells[68].

Seventeen *KIR* genes have been identified and are found clustered in a head to tail fashion covering 150 kb of 19q13.4[69]. Only fourteen of these are expressed and the other three are pseudogenes. Based on gene content in the KIR cluster, over a hundred different profiles have been identified[69], with not all genes being present in all individuals. These haplotypes derive from two ancestral haplotypes, designated A and B, with A having mostly inhibitory *KIR*s and B having more activating receptors. There are, however, many subtypes of these haplotypes with various combinations of these seventeen genes. Many groups are currently attempting to elucidate the role of *KIR* in reproduction as these genes are thought to play a role in recurrent spontaneous abortion as well as pre-eclampsia[70]. These genes made interesting candidates for molar pregnancies due to their role in HLA recognition.

The HLA system has long been suspected to play a role in the aetiology of molar pregnancies, with many groups investigating the link between HLA sharing between couples and their risk of gestational trophoblastic diseases[71-75]. However, these studies have offered conflicting results, with some documenting an increased HLA sharing[72], while others found GTD to be more common when couples were incompatible at several loci[74]. Other studies found no correlation, neither negative nor positive[75], and two groups found a higher frequency of a specific HLA antigen to be associated with moles or GTD[71,73]. All of the expressed genes in the KIR cluster were screened in our patients and no mutations were found. However, all of our familial patients, and for one sporadic case analysed, are homozygous for their KIR haplotypes and, as the *KIR*s are highly polymorphic both at the allelic and haplotypic levels, this is rare and interesting even of itself. So, while none of the *KIR* genes are the direct cause of hydatidiform moles, they

may affect the subsequent sequelae. Their homozygosity for the haplotypes may limit how the women may recognise and respond to the mole once it has formed, and may play a role in how long the mole persists and whether or not it becomes invasive or metastatic. This is an area that would be worth analysing in greater depth.

### *NALP7*

The 1.1 Mb region contained seven members of the NALP family, including *NALP5*, also known as *MATER* (Maternal Antigen That Embryos Require). Initially, *MATER* was considered an excellent candidate gene for molar pregnancy as it has been shown in mice that the lack of this gene causes embryos to arrest at the two cell stage[76]. This gene, and indeed all seven NALPs in the region, had been screened and eliminated as the causative gene. Recently, another group reported that they too had screened *NALP5* in women with recurrent moles and likewise found no mutations[58]. For these reasons, though the extended candidate region included an additional NALP gene, *NALP7*, we felt the *KIR* genes were far stronger candidates and proceeded with screening them first, a task that took the better part of a year to complete. While I was in the process of screening the KIR genes, Rabia Kahn, a summer student performing an independent study project, came to our lab and was given the task of screening *NALP7*. Since I had started my work on the KIR, new information had come to light concerning the role *NALP7*. It had been implicated in testicular seminomas[77] and had been shown to be a regulator of IL-1β[78].

*NALP7*, belongs to a newly recognized family of cytoplasmic proteins involved in the innate immune system[79]. This family is characterized by the presence in their amino terminal of a PYRIN domain, which acts in protein-protein interactions. Fourteen members of this family have been identified and, except for NALPs 1 and 3, which are on

chromosomes 17 and 1 respectively, the genes for NALPs can be found in two clusters on chromosomes 11q15 and 19q13.4, with the latter containing nine members of the family. Members of the NALP family are thought to play a role in inflammation and indeed mutations in one of the NALPs (*NALP3*) lead to Muckle-Wells Syndrome (OMIM 191900) and Familial Cold Autoinflammatory Syndrome (OMIM 120100). Both are hereditary autoinflammatory diseases[79].

A cDNA microarray assay has recently identified NALP7 as being upregulated in testicular seminoma cells[77]. Three splice variants of this gene were identified in normal tissue: V1 to V3. Only one variant, V1, which lacks exon 10, was shown to have increased expression in the tumour cells. This indicates that different splice forms may play different physiological roles. Expression was also identified in embryonal carcinoma cells. Treatment of these cells with *NALP7* siRNA decreased the amount of NALP7 and suppressed the growth of these tumours, thus pointing to a possible role of NALP7 in cell proliferation and tumour growth. Furthermore, analysis of NALP7 at the protein level has shown it to interact with IL-1β and caspase-1[78]. This is of particular interest because, while IL-1β is a cytokine important in the immune response to pathogens, it is also known to play an important role in embryonic implantation. IL-1β and its receptors are expressed by the embryo and the maternal reproductive tract and allow signalling between them prior to implantation. Also, mouse studies have shown that antagonizing the IL-1 receptor prevents implantation.[2]. However, implantation is not the only stage in female reproduction where IL-1β is necessary; it has also been found to act earlier playing a role in ovulation and oocyte maturation[80]. The Caspase family contains proteins known to be key players in the apoptosis pathways. The main function

of caspase-1, however, is in the inflammatory process where, upon stimulation by LPS, it processes pro-IL-1β into its active form so that it can be secreted. Caspase-1 is not thought to act directly in apoptosis although its overexpression has been shown to promote apoptosis[81]. Caspase-1 is also is initially expressed as an inactive precursor, procaspase-1, which must be cleaved to be activated[81]. NALP7 interacts with both precursor proteins, pro-IL-1β and procaspase-1, and prevents their processing into their active forms. Thus NALP7 acts as an inhibitor of IL-1β and IL-1β, in turn, induces expression NALP7, forming a negative feedback loop. It follows logically and has been shown experimentally that NALP7 expression is, therefore, also induced by LPS[78].

With this current understanding of *NALP7*, its roles in inflammation and tumour formation, we can see that this gene is indeed an excellent candidate for molar pregnancies. This gene was screened and homozygous mutations were found in four families of different ethnic origin: German, Lebanese, Pakistani and Indian. Additionally, we screened sporadic cases whose linkage to chromosome 19 was undefined and found two heterozygous mutations in one of these individuals. The discovery of these mutations and their description can be found in the accompanying manuscript.

# INTRODUCTION TO THE MANUSCRIPT

With a causative locus for familial moles having been mapped to chromosome 19q13.4, we undertook a candidate gene approach to identify the defective gene in this region. We screened all of the genes in the 1.1 Mb candidate region as described by Hodges et al.[57] Having found no mutations in these genes, we re-evaluated the candidate region. Taking into consideration our previously mentioned doubts about the proximal border defined by Sensi[56], we decided to move the border back to that based on our Lebanese family and screened the additional genes: *NCR1*, *FCAR*, the fourteen expressed genes of the *KIR* gene cluster and *NALP7*. In total seventy-seven known or predicted genes where screened; I screened twenty-three of these as listed in Table 1.

**TABLE 1: LIST OF GENES SCREENED**

| Known Genes | Predicted Genes |
|---|---|
| RDH13 | LOC352909 |
| NALP8 | BC040925 |
| NALP9 | JASOR |
| NCR1 | AK09618 |
| FCAR | BC034929 |
| ZNF579 | |
| KIR3DL2 | |
| KIR2DL4 | |
| KIR2DL3 | |
| KIR2DL2 | |
| KIR2DL1 | |
| KIR3DL1 | |
| KIR3DS1 | |
| KIR2DS4 | |
| KIR3DL3 | |
| KIR2DL5 | |
| KIR2DS1 | |
| KIR2DS5 | |

We also analysed a new family with recurrent moles, MoPa61, which significantly narrowed down the candidate region to 0.65 Mb.

The following manuscript, entitled "*NALP7*, a maternal effect gene responsible for recurrent hydatidiform moles and reproductive wastage," describes the discovery of mutations in the gene *NALP7* in our patients affected with familial moles, and thus identifying it as the causative gene for the disorder in these people. Two of the mutations we identified affect invariant splice sites; therefore, we also investigated, via RT-PCR and sequencing, the effect of these mutations on the splicing of this gene and their consequences at the protein level.

The techniques described in the paper were the same used to screen and exclude the rest of the genes in the candidate region.

This manuscript was accepted for publication in Nature Genetics in November 2005.

# CONTRIBUTION OF THE AUTHORS

**Sharlene Murdoch**

- Performed mutation screening and excluded twenty four genes in the candidate region
- Trained and supervised Rabia in the design of *NALP7* primers and screening the gene in patients
- Screened controls for the presence of two mutations in *NALP7*
- Determined the segregation of two mutations in their respective families
- Extracted RNA from endometrial tissues
- Performed RT-PCRs to determine *NALP7* expression in uterus, ovary and endometrium and to assess the effect of the two splice mutations
- Screened some recurrent cases for mutations in *NALP7*
- Constructed some of the figures
- Participated in editing of the manuscript

**Ugljesa Djuric**

- Performed Genotyping of MoIn68 and MoIn69.

**Batool Mazhar and Bhawna Ratti**

- Provided us with blood samples from all members of family MoPa61

**Rabia Khan**

- Performed mutation analysis of *NALP7*
- Screened controls for two of the mutations and determined their segregation in the families
- Screened four recurrent cases for mutations
- Participated in construction of figures

**Samir Hanash and Rork Kuick**

- Performed Restriction Landmark Genome Scan

**Rashmi Bagga**

- Provided us with DNA from families MoIn68 and MoIn69

**Renate Kircheisen**

- Provided us with DNA from all family members of MoGe2, and twice provided us with fresh blood from two of the patients

**Guy A. Rouleau**

- Provided our group with unconditional support and help that were essential and critical to speed up our search for the causative gene

**Muheiddine Seoud**

- Provided us with DNA from all members of family MoLb1
- Gave us fresh molar tissue and paraffin blocks from all their moles
- Sent us fresh blood samples from the patients, three times

**Rima Slim**

- Supervised project
- Wrote and edited manuscript

# Mutations in *NALP7*, a maternal effect gene, result in recurrent hydatidiform moles and reproductive wastage in humans

Sharlene Murdoch[1,2], Ugljesa Djuric[1,2], Batool Mazhar[3,9], Muheiddine Seoud[4,9] , Rabia Khan[1,2], Rork Kuick[5], Rashmi Bagga[6], Renate Kircheisen[7], Asangla Ao[2], Bhawna Ratti[3], Samir Hanash[5], Guy A Rouleau[8], Rima Slim[1,2]

[1]Departments of Human Genetics and [2]Obstetrics and Gynecology, McGill University Health Center, Montreal H3G 1A4, Canada

[3]Dept of Obstetrics and Gynecology, Maternal & Child Health Centre Unit-II, Pakistan Institute of Medical Sciences, Islamabad, Pakistan

[4]Dept of Obstetrics & Gynecology, American University of Beirut, P.O. Box 11-236, Lebanon

[5]Dept of Pediatrics and Communicable Diseases, University of Michigan, Ann Arbor 48109, USA

[6]Dept of Obstetrics & Gynecology, Post Graduate Institute of Medical Education and Research (Pgimer), Chandigarh 160012, India

[7]Institut für Klinische Genetik, Mainz 55131, Germany

[8]Center for the Study of Brain Diseases, Notre Dame Hospital, Montreal H2L 4M1, Canada

[9]These authors contributed equally to this work

**Abstract**

Hydatidiform mole (HM) is an abnormal human pregnancy with no embryo and cystic degeneration of placental villi. We report five mutations in the maternal gene, *NALP7*, in patients with familial and recurrent HMs. NALP7 is a member of the CATERPILLER protein family involved in inflammation and apoptosis. *NALP7* is the first maternal effect gene identified in humans and is also responsible for recurrent spontaneous abortions, stillbirths, and intrauterine growth retardation.

Recurrent hydatidiform moles (HMs) is a rare clinical entity in which molar tissues are diploid and have a biparental contribution to their genome. We previously mapped a maternal recessive locus responsible for familial HMs to 19q13.4[1]. To identify the defective gene, we undertook a candidate gene approach and screened all the genes present in the 1.1 Mb minimal interval[2]. However, we did not find any mutations. We therefore relied on our families, refined the mapping of the proximal boundary in MoLb1, and genotyped an additional family, MoPa61[3], at 19q13.4 markers (Supplementary Fig. 1 and 2). Based on data from MoLb1 and MoPa61, we fine mapped the HM candidate region to 0.65 Mb between 11515-31 and COX6B2ex3.

By screening the additional genes added at the proximal boundary, we identified in *NALP7* two different mutations affecting the invariant G of the GT splice donor site at the junction of exon 3/intron 3 (IVS3+1G>A) in a patient from MoLb1 (Fig. 1a) and exon 7/intron 7 (IVS7+1G>A) in a patient from MoPa61 (Fig. 1c). Both mutations cosegregated with the disease phenotype in their respective families (Fig. 1b) and were not found on 200 control chromosomes from women of various ethnic groups

(Supplementary Table 1). Screening of two additional families, MoGe2 and MoIn68[4], and a single family member with recurrent moles, MoIn69-2 identified three amino acid substitutions R693W, R693P, and N913S (Table 1). Amino acid Arg693 is a conserved residue in chimpanzee and cow *NALP7* as well as in human, cow, and dog *NALP2*. The three substitutions cosegregated appropriately in each family and were not present on 348 control chromosomes. These results establish *NALP7* as the causative gene for recurrent hydatidiform moles and various forms of reproductive wastage.

*NALP7* is expressed in a wide variety of normal[5] and pathological tissues (Unigene http://www.ncbi.nlm.nih.gov/UniGene and the Gene Expression Omnibus http://www.ncbi.nlm.nih.gov/geo/). It consists of 11 exons encoding a 1009 amino acid protein (the longest isoform). Three transcriptional isoforms *NALP7*V1-V3 with alternative splicing involving exons 5, 9, and 10 have been described[6]. To investigate the role of *NALP7* in the pathology of moles, a disease caused by a maternal defective gene, we checked its transcription by RT-PCR in normal human uterus, endometrium, and ovary. We identified two *NALP7* transcripts, V1 and V2, in uterus and ovary, but only V1 in endometrium. Using two round PCR, we also identified *NALP7* transcripts in unfertilized denuded oocytes at the germinal vesicle and metaphase I stages as well as early cleavage embryos at four to eight cells.

We investigated the effect of the two invariant splice mutations using RT-PCR on RNA from EBV-transformed lymphoblastoid cell lines from two patients from MoLb1 and three patients from MoPa61. We demonstrate that IVS3+1G>A results in two splice isoforms, one leading to the inclusion of the first 4 bp of intron 3 in the RNA between exons 3 and 4 (Figure 1a) and results in the addition of two amino acids followed by a

stop codon, TGA, and one to the exclusion of exon 3. We note that a splice isoform without exon 3 was also observed at a very low amount in controls and is present in one EST (BF768507). IVS7+1G>A leads to the inclusion of the entire intron 7, which is expected to add next to exon 7 only one amino acid followed by a stop codon, TAA (Figure 1c-d).

NALP7 is one of 14 NALP proteins, a large subfamily of the CATERPILLER protein family involved in intracellular regulation of bacterial-induced inflammation. NALP7 protein contains an amino-terminal PYRIN domain (PYD) (also called DAPIN), a putative protein-protein interaction domain found in all the CATERPILLER proteins and is thought to function in apoptotic and inflammatory signaling pathways; a NACHT domain found in neuronal apoptosis inhibitor proteins as well as in those involved in major histocompatibility complex (MHC) class II transactivation and caspase-recruitment proteins; a nuclear localization signal (NLS) present within the NACHT domain; and 9 to 10 leucine-rich repeats (LRRs) (depending on the splicing isoforms) found in the Ran GTPase activating proteins (RanGAP1), highly conserved proteins essential for nuclear transport, cell cycle regulation, mitotic spindle formation, and post mitotic nuclear envelope assembly[7]. NALP7 has been shown *in vitro* to inhibit caspase-1 dependent IL-1β secretion, which in turn induces NALP7 expression[5]. *NALP7* is upregulated in testicular seminoma tumors where its down regulation by transfection with small interfering RNA results in growth suppression[6]. To date, only one NALP gene, *NALP3*, has been found responsible for human diseases. Mutations within *NALP3* lead to three different diseases, familial cold autoinflammatory syndrome (FCAS/FCU), chronic infantile neurologic cutaneous and articular (CINCA) syndrome, Muckle-Wells

syndrome (MWS), all involving multisystemic inflammation. In mice, one *Nalp* gene, *Nalp5*, has been shown to be required for embryonic development beyond the two-cell stage[8]. Two other NALPs, 9 and 14, show oocyte restricted expression and are believed to play a role in reproduction. No mouse ortholog of *NALP7* has been identified. *NALP7* is believed to have originated from mouse *Nalp2* by gene duplication following speciation.

NALP7 protein does not contain any of the known domains found in DNA methyltransferases or any DNA binding domain, it seems therefore unlikely that it plays a direct role in the establishment or the maintenance of CpG methylation. The abnormal DNA methylation observed so far only at imprinted genes in molar tissues could be a consequence of a defective oocyte growth and/or maturation, during which maternal methylation marks are added. We note that patients with *NALP7* mutations ovulated normally without any treatment and four of them had nine to fifteen different conceptions that ended up in molar pregnancies or various forms of reproductive wastage, which is unlikely to occur if NALP7 is needed in the oocytes before fertilization.

NALP7 is a negative regulator of IL-1β, a pleiotropic cytokine that activates a number of immunological and inflammatory pathways. IL-1β is abundant in the uterine milieu during the peri-implantation period where it facilitates the implantation of the blastocyst, regulates the protease network, and controls the extent to which the trophoblast may invade the maternal endometrium[9, 10]. Inflammation is a complex pathway, although it may be triggered by one defective gene, modifier genes as well as environmental factors may modulate the intensity and extent of the resultant inflammatory response. The deregulation of the inflammatory pathways in the

reproductive tract or the uterus in women with defects in *NALP7* would explain the wide variability in the phenotype of their conceptuses and late spontaneous abortions, stillbirths, and normal pregnancies with intra-uterine growth defect. Overexpression of NALP7, in vitro, inhibits IL-1β production upon stimulation with lipopolysaccharide (LPS), a component of the cell walls of gram-negative bacteria known to cause fetal loss in humans and animals. In humans, genital tract infections by gram-negative bacteria are among the major causes of infertility, ectopic pregnancy, abortion, and preterm labor[11]. The mechanism by which LPS induces abortions in mice involves a marked inflammatory response that is mediated by the activation of macrophages and the release of a number of cytokines that result in increased apoptosis[12] and nitric oxide production[13]. Both apoptosis[14] and nitric oxide[15] are significantly elevated in women with the common form of HMs. This suggests that the common forms of HMs, although they may not be due to mutations in *NALP7*, may result from the deregulation of the same inflammatory pathway. The identity of the causative gene for recurrent HMs pinpoints an important pathway that may underlie the etiology of non-recurrent moles and is highly relevant to the large number of patients with this condition.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Supplementary Information accompanies the paper

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**Competing interests statement** The authors declare that they have no competing financial interests.

**Correspondence** and request for materials should be addressed to R.S. (rima.slim@muhc.mcgill.ca).

**Figure 1**

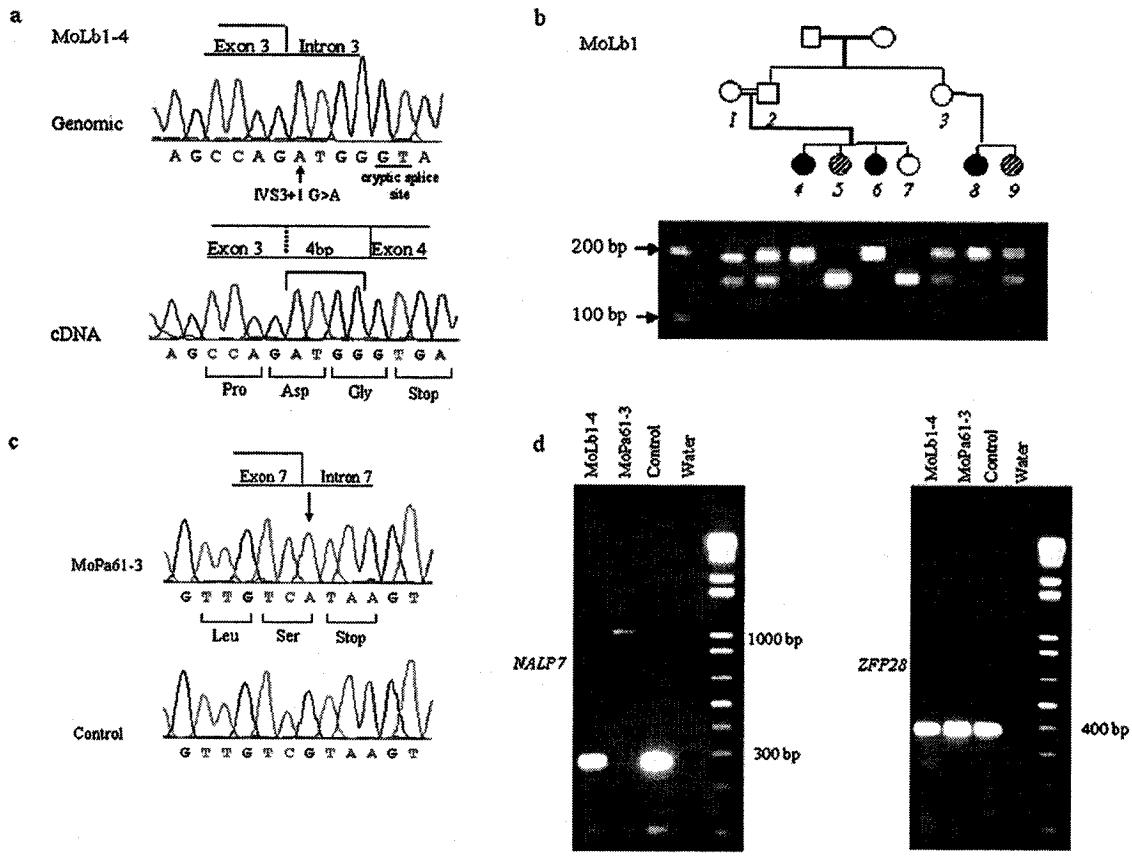

a
MoLb1-4
Exon 3
Intron 3
Genomic
A G C C A G A T G G G T A
IVS3+1 G>A
cryptic splice site
Exon 3
4bp
Exon 4
cDNA
A G C C A G A T G G G T G A
Pro
Asp
Gly
Stop
b
MoLb1
1
2
3
4
5
6
7
8
9
200 bp
100 bp
c
Exon 7
Intron 7
MoPa61-3
G T T G T C A T A A G T
Leu
Ser
Stop
Control
G T T G T C G T A A G T
d
MoLb1-4
MoPa61-3
Control
Water
NALP7
1000 bp
300 bp
ZFP28
400 bp

**Figure 1**

Analysis of splice site mutations. (a) sequence trace indicating IVS3+1G>A and its effect on the cDNA. The resultant cDNA has an additional 4 bp, which lead to a premature stop codon downstream of exon 3. (b) segregation of IVS3+1G>A in MoLb1. The mutation removes a *Bst*N1 restriction site resulting in a 206bp band; a 153 bp band indicates presence of the normal allele. Black symbols indicate affected women, white symbols unaffected, and shaded symbol indicates women with unknown disease status. (c) sequence trace indicating IVS7+1G>A in patient MoPa61-3. This mutation leads to the addition of one amino acid followed by a stop codon. (d) RT-PCR with primers in NALP7 exons 6 and 8 showing a 1034 bp band in MoPa61-3 resulting from the inclusion of the entire intron 7. The *ZFP28* gene was amplified on the same samples to show the presence of RNA in all samples.

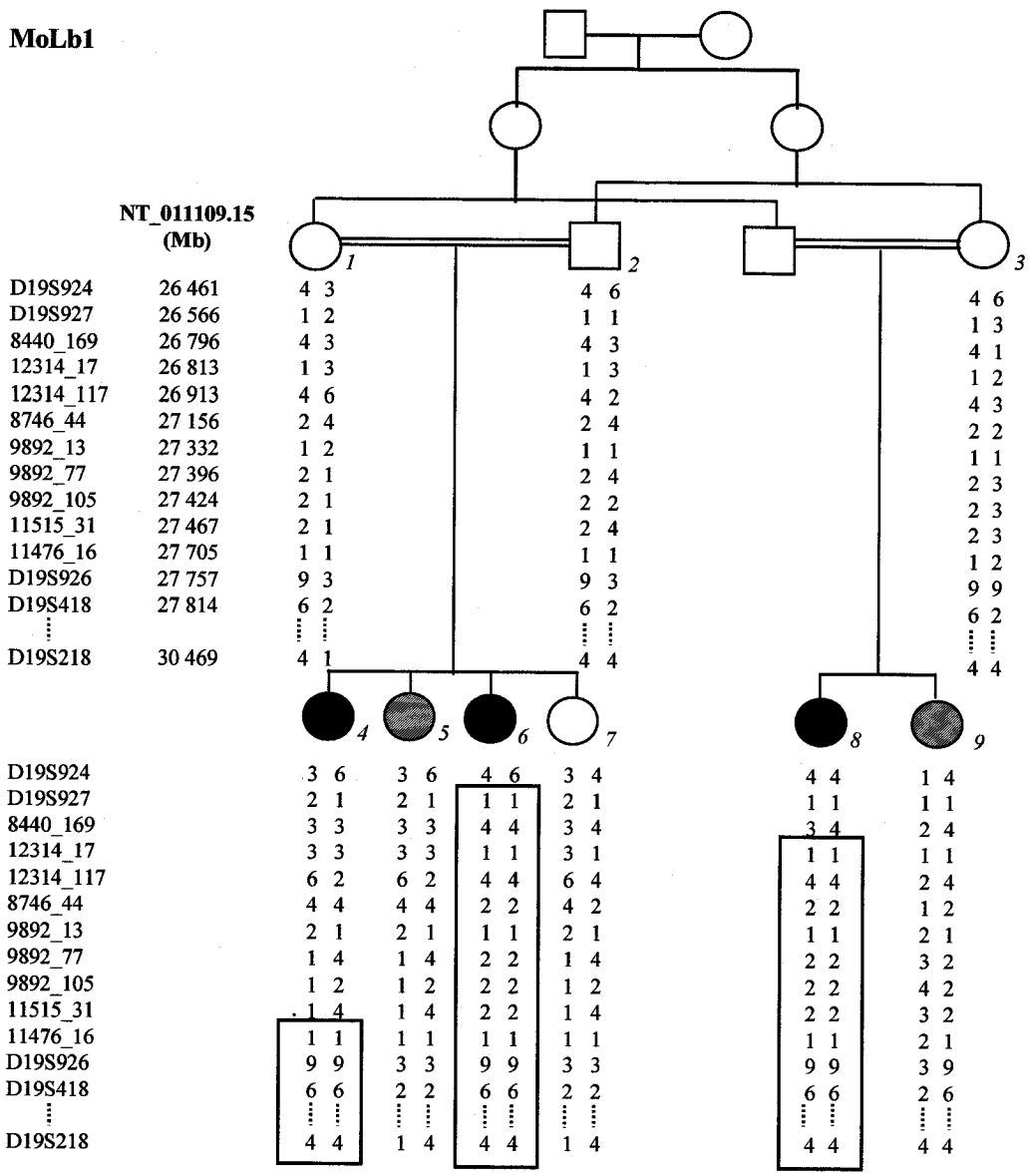
**Table 1 Summary of mutation analysis, ethnic origin, and pregnancy outcomes**

| Family | Population | Location | Nucleotide change | Amino acid change | Pregnancy outcomes | Reference |
|---|---|---|---|---|---|---|
| Familial cases of recurrent moles | | | | | | |
| MoLb1[a] | Lebanese | Intron 3 | IVS3+1G>A | | NP[c], SB, SA, CHM, PHM, PTD | 1 |
| MoPa61[a] | Pakistani | Intron 7 | IVS7+1G>A | | SA, CHM | 3 |
| MoGe2[a] | German | Exon 5 | 2077C>T | R693W | CHM | 1 |
| MoIn68[a] | Indian | Exon 5 | 2078G>C | R693P | SB, CHM | 4 |
| Single family member with recurrent moles | | | | | | |
| MoIn69-2[b] | Indian | Exon 5<br>Exon 9 | 2078G>C<br>2738A>G | R693P<br>N913S | CHM, IM | present study |

SB, stillbirth; SA, spontaneous abortion (7-20 weeks); CHM, complete hydatidiform mole, PHM, partial hydatidiform mole; PTD, persistent trophoblastic disease; IM, invasive mole; NP, normal pregnancy. Nucleotide positions are given according to RefSeq mRNA NM_206828, amino acid positions according to Q8WX94. [a]Homozygous; [b]compound heterozygous; [c]two normal term deliveries leading to normal healthy adults, in one of them the baby had severe intrauterine growth retardation (1.6 kg at birth).

## Supplementary Figure 1

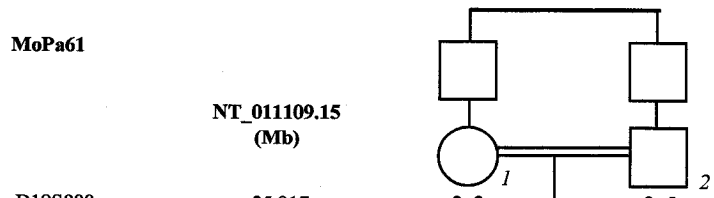
**MoLb1**



**Supplementary Figure 1**

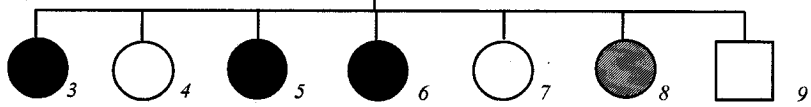
Partial pedigree of family MoLb1 showing the limit of the proximal boundary of the hydatidiform mole candidate locus on 19q13.4. Markers are ordered (top to bottom) from centromere to telomere and their positions are given in contig NT_011109.15. Genotyping was performed using publicly available and newly generated microsatellite markers (deposited to Genbank) by incorporation of radiolabelled nucleotides in the PCR amplification and separation of the products on 5% denaturing polyacrylamide gels. The black box shows the region that is homozygous in each patient. The proximal boarder of the candidate region is defined by marker 11515_31 due to its heterozygosity in patient 4.

## Supplementary Figure 2




**MoPa61**

| | NT_011109.15 (Mb) | 1 | 2 |
|---|---|---|---|
| D19S888 | 25 917 | 2 2 | 2 5 |
| D19S921 | 26 039 | 3 6 | 4 3 |
| D19S572 | 26 373 | 1 7 | 1 1 |
| D19S927 | 26 566 | 4 2 | 1 4 |
| 12314-117 | 26 913 | 3 2 | 1 3 |
| 9892-77 | 27 396 | 5 3 | 2 5 |
| D19S926 | 27 757 | 6 1 | 6 6 |
| FLJ20510ex13cd_del | 27 780 | 1 2 | 1 1 |
| D19S418 | 27 814 | 2 6 | 4 2 |
| D19S880 | 27 950 | 1 2 | 2 1 |
| D19S605 | 28 020 | 4 4 | 2 4 |
| BV102459 | 28 087 | 2 3 | 2 2 |
| D19S877 | 28 108 | 8 4 | 3 8 |
| COX6B2 ex3 | 28 133 | 1 2 | 2 2 |
| UBE2S | 28 181 | 1 2 | 2 2 |
| KLP1 | 28 265 | 1 1 | 2 2 |
| SRCR5'a2b | 28 270 | 2 2 | 1 1 |
| D19S891 | 28 301 | 4 2 | 6 1 |
| BV102467 | 28 341 | 2 1 | 1 2 |
| BV102464 | 28 362 | 3 3 | 4 2 |
| BV102465 | 28 409 | 4 5 | 2 2 |
| PYAF6r12 | 28 565 | 2 3 | 1 2 |
| D19S210 | 29 287 | 4 4 | 4 1 |




| | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|
| D19S888 | 2 5 | 2 2 | 2 5 | 2 5 | 2 2 | 2 2 |
| D19S921 | 3 3 | 3 4 | 3 3 | 3 3 | 3 4 | 3 4 |
| D19S572 | 1 1 | 1 1 | 1 1 | 1 1 | 1 1 | 1 1 |
| D19S927 | 4 4 | 4 1 | 4 4 | 4 4 | 4 1 | 4 1 |
| 12314-117 | 3 3 | 3 1 | 3 3 | 3 3 | 3 1 | 3 1 |
| 9892-77 | 5 5 | 5 2 | 5 5 | 5 5 | 5 2 | 5 2 |
| D19S926 | 6 6 | 6 6 | 6 6 | 6 6 | 6 6 | 6 6 |
| FLJ20510ex13cd | 1 1 | 1 1 | 1 1 | 1 1 | 1 1 | 1 1 |
| D19S418 | 2 2 | 2 4 | 2 2 | 2 2 | 2 4 | 2 4 |
| D19S880 | 1 1 | 1 2 | 1 1 | 1 1 | 1 2 | 1 2 |
| D19S605 | 4 4 | 4 2 | 4 4 | 4 4 | 4 2 | 4 2 |
| BV102459 | 2 2 | 2 2 | 2 2 | 2 2 | 2 2 | 2 2 |
| D19S877 | 8 8 | 8 3 | 8 8 | 8 8 | 8 3 | 8 3 |
| COX6B2 ex3 | 1 2 | 1 2 | 1 2 | 1 2 | 1 2 | 1 2 |
| UBE2S | 1 2 | 1 2 | 1 2 | 1 2 | 1 2 | 1 2 |
| KLP1 | 1 2 | 1 2 | 1 2 | 1 2 | 1 2 | 1 2 |
| SRCR5'a2b | 2 1 | 2 1 | 2 1 | 2 1 | 2 1 | 2 1 |
| D19S891 | 4 1 | 4 6 | 4 1 | 4 1 | 4 6 | 4 6 |
| BV102467 | 2 2 | 2 1 | 2 2 | 2 2 | 2 1 | 2 1 |
| BV102464 | 3 2 | 3 4 | 3 2 | 3 2 | 3 4 | 3 4 |
| BV102465 | 4 2 | 4 2 | 4 2 | 4 2 | 4 2 | 4 2 |
| PYAF6r12 | 2 2 | 2 1 | 2 2 | 2 2 | 2 1 | 2 1 |
| D19S210 | 4 1 | 4 4 | 4 1 | 4 1 | 4 4 | 4 4 |

**Supplementary Figure 2**

Pedigree of family MoPa61 with recurrent hydatidiform moles. Twenty-three informative microsatellite markers were genotyped to determine linkage to 19q13.4 Markers are ordered (top to bottom) from centromere to telomere and are indicated on the left along with their position in contig NT_011109.15. Black symbols indicate affected women, white symbols unaffected, and shaded symbol indicates a woman with unknown disease status. The homozygous region in the three affected sisters is indicated. These data define marker COX6B2 as the distal boundary of the HM candidate region due to its lack of homozygosity in all three affected sisters.

## Supplementary Table 1

**Ethnic origin of normal control samples**

| Origin | Number | Description |
|---|---|---|
| CEPH families of European descent | 43 | unrelated women with 5 to 16 pregnancies |
| Mostly of European descent (from families with various diseases collected from Montreal | 107 | unrelated women with 5 to 16 pregnancies |
| Lebanese | 40 | unrelated women with 5 to 16 pregnancies |
| Pakistani | 40 | unrelated subjects from the general population |

## Supplementary Methods

### Patients

Patient consent was obtained according to the institutional review board committees at the participating institutes. DNA was extracted from total blood or from Epstein-Barr virus-transformed lymphocytes by standard methods. DNA from molar and normal chorionic villi was extracted from fresh tissues. Total RNA from normal human ovary (single female 30 years) and uterus (pooled from 3 females aged 40-61) were purchased from Stratagene and BD Biosciences, respectively. Total RNA from normal endometrium was obtained from three patients with various gynaecological disorders, but normal endometrium. The tissues were collected and dissected in RNALater (Ambion) and stored at –80°C until RNA extraction. Unfertilized denuded oocytes and early cleavage embryos were collected as previously described in lysis buffer and stored frozen until use.

### Mutation screening and analysis

Genomic structure of the screened genes were obtained from publicly available databases (http://genome.ucsc.edu/) and the primers flanking predicted exons, exon/intron boundaries and 5'and 3'UTRs were designed using Primer Select v5.05 (DNAStar) (The primer sequences and PCR conditions are available upon request). Exons were PCR amplified, visualized on 2% agarose gels stained with ethidium bromide, and sequenced directly at Genome Quebec using a 3730XL DNA Analysis System (Applied Biosystems). Sequences were aligned using SeqManII v5.05 and screened for mutations.

**RT-PCR**

Total RNAs from various tissues were extracted using Trizol (Invitrogen) according to standard conditions. Two rounds of PCR were performed on two independent sets of oocytes (2 to 3 each) and single embryos. Primer sequences and PCR conditions are available upon request. Sequencing of cDNA fragments were done on direct PCR products or after gel purification of the appropriate bands and cloning using the TOPO TA Cloning Kit (Invitrogen).

# CONCLUSIONS

Hydatidiform moles are a serious, potentially premalignant condition about whose origins much is hypothesised but little is definitively known. This condition affects women of all ages and of all walks of life and can cause much grief and heartache for those wishing to conceive and bear a child. The knowledge that molar pregnancies can run in families has given much insight into this disorder and opened up a new area of investigation. As with many disorders, by studying the familial form we can isolate a gene responsible thereby elucidating the pathway leading to its pathogenesis. This analysis holds potential not only for the affected families but for those women with sporadic moles as well, for logic would follow that the defective pathway in familial moles may be the same as that for all moles, though the damaged component may be different in different cases. The first step to determining treatment for any condition is, of course, determining its cause.

Throughout this work I have described what is known and what is hypothesised about hydatidiform molar pregnancies based on the empirical data and on comparison with similar conditions. Over the years, a vast amount of data has been collected on moles and the women affected with them in an attempt to elucidate its aetiology, but without identification of a gene it has all remained speculative. Here I have presented our findings that *NALP7*, a gene involved in the inflammatory process and whose downstream effects could include alteration of DNA methylation, is the defective gene in those families linked to chromosome 19q13.4. The discovery of mutations in this gene in some sporadic cases of recurrent moles implicates *NALP7* as a major gene for this pathology.

In two of our patients we observed splice changes which lead to the addition of a premature stop codon. Identification of splice mutations is in line with what was observed in testicular seminomas, indicating that different splice variants may play important physiological roles[77]. Mutations that disrupt proper splicing have been identified in many diseases including Frasier syndrome (OMIM 136680), which is caused by abolishment of an alternate splice site in exon 9 of the Wilms tumour suppressor gene; and Atypical Cystic Fibrosis (OMIM 602421), caused by a polymorphism that leads to the skipping of exon 9 of the *CFTR* gene[82]. Splice site mutations can lead to a variety of changes in the mRNA including skipping of the affected exon, inclusion of the affected intron, or the use of an alternate cryptic splice site. All of these may lead to a change in reading frame of the mRNA, thus altering the resulting protein[82]. It is estimated that 15% of disease causing point mutations do so by disrupting normal splicing. This number, however, is believed to be underestimated, as it is now thought that many missense, exonic and intronic variants also lead to aberrant splicing[82].

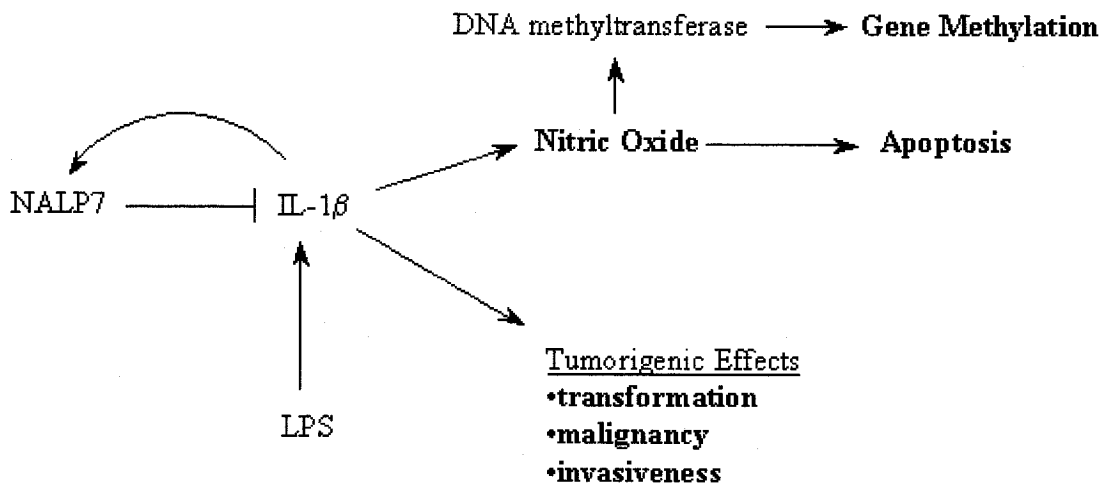
At this time little is known about the normal physiological role of *NALP7*; it is known to play a role in inflammation, cell proliferation, and, possibly tumorigenesis[77]. It is known to interact with IL-1β and caspase-1[78], both thought to be involved in processes important during embryonic implantation (inflammation and apoptosis[5], respectively). Presently, *NALP7*'s link to DNA methylation is tenuous. Although it is known to interact with IL-1β, which has, in turn, been shown to induce gene silencing through DNA methylation[83], no direct effect of *NALP7* on methylation has been demonstrated; nevertheless it is interesting to speculate about a link between defect in *NALP7* and the abnormal methylation seen in moles. Furthermore, while many contemporary theories

peg abnormal imprinting as the cause of molar pregnancies, our data (not shown) indicate normal X-inactivation and normal methylation of LINE elements are occurring in moles, which shows that postzygotic demethylation and remethylation are occurring normally. This may suggest that the disregulation of methylation observed in moles may be a downstream effect of the molar phenotype rather than a cause.

Thus, *NALP7* fits very well as the causative gene for hydatidiform moles. Many correlations can be found between what is known about the action of the gene and the clinical findings of molar pregnancies (figure 3). Nitric oxide has been shown to be increased in the plasma of women with complete moles[84] and IL-1β is known to induce nitric oxide production[83]. Nitric oxide, in turn, can promote apoptosis[85], which has likewise been demonstrated to be increased in hydatidiform moles[6-8]. Interestingly expression of bcl-2, an anti-apoptotic protein, has also been shown to be increased in complete moles[86]. This may indicate a struggle occurring in this tissue between apoptosis and the need to counteract it, similar to the previously mentioned balancing act between proliferation and apoptosis hypothesised for pre-eclampsia[5]. IL-1β has also been implicated in the malignant transformation of tumour cells as it is thought to play a role in invasiveness and metastasis[87]. In certain types of leukaemia (Acute Myelogenous Leukaemia and Chronic Myelogenous Leukaemia) inhibitors of interleukin-1 have been shown to suppress tumour cell growth. IL-1β is also expressed in breast cancers, where its level of expression correlates with tumour aggressiveness. Conversely, anti-tumorigenic effects of IL-1β resulting in reduced growth and metastasis have also been noted[87], suggesting a need for tight regulation of IL-1β production. Furthermore, IL-1β is one of the pro-inflammatory cytokines induced by LPS stimulation, which, as

previously mentioned is known to have adverse affects on implantation and trophoblast development and to cause spontaneous abortions[11].

**FIGURE 3: HYPOTHETICAL ROLE OF NALP7 IN HYDATIDIFORM MOLES**




**Possible role of NALP7.** This figure summarizes what is known about the physiological role of NALP7 (its regulation of IL-1β) and how it may be linked to some of the clinical findings of hydatidiform moles. In bold are the processes known to be associated with moles

Identification of the gene is just the first step in understanding the pathological process leading to moles, but a crucial one nonetheless. It points to the pathway involved and the challenge now is to determine how mutations in this gene lead to the complex phenotype that is the hydatidiform mole. As mentioned, many of the clinical findings of molar pregnancies can be explained by the action of NALP7 through its effect on IL-1β; however, as was also mentioned, IL-1β acts in many processes of female reproduction such as ovulation, oocyte maturation and implantation. This begs the question: which step is key for the formation of moles? The affected women have had

multiple pregnancies, therefore, ovulation is occurring normally and therefore, that step is less likely. The occurrence of stillbirths in these women and indeed the occasion of normal pregnancies in two of them (from MoLb1) may indicate that oocyte maturation is also occurring normally, thus leaving the most likely candidate for the affected pathway to be implantation. Implantation itself is a complex process and it will be important to determine at which stage *NALP7* acts. Furthermore, at such a time when the second locus for this disorder is mapped (a process currently underway for the previously mentioned MoIr56), the identification of defective gene on 19q13.4 will aid in the prioritization of candidate genes in that locus, as the second gene will be expected to act in a similar pathway.

### *Future directions*

The next step is to determine the mechanism leading to the molar phenotype. To do this we must determine two things: the effect of NALP7 mutations on IL-1β secretion and at what point in development proper NALP7 expression is needed to circumvent the formation of moles. The effect of abnormal NALP7 on IL-1β secretion can be evaluated *in vitro*, either by using cells from the patients themselves or by transfection of cells with a construct containing the mutated form of the gene. As discussed above, the logical place action of NALP7 is at implantation and we know that IL-1β plays a role in implantation and that NALP7 mRNA is expressed in normal uterus. Our preliminary data (not shown), however, also found NALP7 mRNA to be expressed in immature oocytes, and blastocysts from two cell to six cell stages. We have also evaluated the expression of NALP7 mRNA in normal human endometrium and found that it does not express the V2 splice variant (not shown). It would be interesting to study, via Real-

Time PCR, whether expression of NALP7 in the endometrium varies at different times of the reproductive cycle. Were we to witness a peak in expression around the time of implantation, it would support a role for NALP7 in this process. Wherever possible we will attempt to obtain human endometrial tissue to study this, however, samples of human reproductive tissue are difficult to acquire; therefore, finding a suitable animal model will greatly enhance our ability to study this phenomenon. The mouse is a commonly used model organism for the study of reproductive diseases, indeed for most human diseases; however, in this case, mice have two drawbacks: the mouse homologue of human *NALP7* is not known and mice have never been reported to have hydatidiform moles. In fact it is hypothesized that the mouse may not have an exact homologue of *NALP7*[78]. Right next to the *NALP7* gene on 19q13.4 lies *NALP2*. These genes are highly similar and are thought to have arisen by gene duplication. *NALP2* (also called *PYPAF2*) is involved in the activation of NF-κB. It has been suggested that the mouse homologue (mouse *Nalp2*) may perform the functions of both human *NALP7* and *NALP2*[78]. This is an interesting theory in that it may also explain why moles are not seen in mice. Mutations in the mouse gene would disrupt both IL-1β secretion and NF-κB activation possibly leading to something more catastrophic than a mole. Moles have been reported, however, in cows[88]. Cow tissues are easier to obtain than human tissues and the bovine *NALP7* homologue is known, rendering investigation of its expression in this animal more feasible. We will obtain bovine endometrial samples from different times in the reproductive cycle, and, if available, tissue from the implantation site itself to examine the expression of NALP7 mRNA. In addition to examining mRNA expression, it would be of interst to know if NALP7 protein is being translated in these tissues. At this time

we cannot say definitively if the crucial role of NALP7 is at implantation because we have also seen its mRNA expressed in oocytes and blastocysts, but if we were to examine protein expression and find that the protein is present in the endometrium but not the oocyte or blastocyst, we could rule those out as the place of action of NALP7. These experiments would necessitate the development of a NALP7 antibody for use in western blots or immunohistochemistry. Two other groups have already developed such an antibody[77,78] and we could obtain the antibody from one of them, or develop our own. Either way these would be important experiments to perform. Mapping the second causative locus and determining the mutated gene therein will also give us another component of the pathway and help us to draw a more detailed picture of the process leading to hydatidiform moles.

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