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(57) **Abrégé/Abstract:**

The non-invasive detection of fetal chromosomal aneuploidies is demonstrated. Alleles of fetal RNA-SNPs present in a biological sample (e.g. maternal blood) containing fetal RNA are detected and quantified in order to determine the ratio of the alleles. This ratio is compared to a standard control consisting of euploid fetuses. Deviation of allele ratio indicates the presence of chromosomal aneuploidy.

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(54) Title: A METHOD FOR THE DETECTION OF CHROMOSOMAL ANEUPLOIDIES

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A METHOD FOR THE DETECTION OF CHROMOSOMAL ANEUPLOIDIES

5 CROSS-REFERENCES TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application No. 60/663,173, filed March 18, 2005.

[0002] NOT APPLICABLE

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BACKGROUND OF THE INVENTION

[0003] Chromosomal aneuploidy is an important cause of morbidity during the prenatal and postnatal life. Assessment of chromosomal aneuploidy has traditionally been associated with the investigation of fetal viability and prenatal diagnosis. Methods for the detection and
15 characterization of chromosomal aneuploidy include karyotyping of metaphase chromosomes, fluorescence in-situ hybridization (FISH) (Homer, J. et al., *Prenat Diagn* **23**:566-571 (2003)), quantitative fluorescence polymerase chain reaction (PCR) (Mann, K. *Methods Mol Med* **92**:141-156 (2004)), gene dosage PCR (Zimmermann, B. et al., *Clin Chem* **48**:362-363 (2002), and array-based comparative genomic hybridization (CGH) (Hu, D.G. et al., *Mol*
20 *Hum Reprod* (2004)).

[0004] Fetal chromosomal aneuploidy is known to contribute significantly to fetal loss and accounts for 50% of the first trimester spontaneous abortions (Chitty, L. *Br Med Bull* **54**:839-856 (1998)). Viable fetuses are especially associated with certain types of chromosomal aneuploidy. Trisomy 21, or Down syndrome, is the most common autosomal aneuploidy
25 compatible with postnatal survival with an occurrence of 1 in 800 live births (Hook, E. B. *Lancet* **2**:169-172 (1981)). Trisomy 21 is the most common reason why couples opt for prenatal diagnosis. At present, definitive diagnosis of a trisomy 21 fetus and other chromosomal aneuploidies rely on the genetic analysis of fetal genetic material obtained by invasive procedures such as amniocentesis and chorionic villus sampling (CVS). By virtue of
30 their invasive nature, these procedures are associated with a finite risk of spontaneous abortion. Hence, other noninvasive approaches have been developed to stratify pregnancies according to the risk of carrying a trisomy 21 fetus. Only pregnancies with a substantive risk,

defined as a risk greater than the procedure-related risk of fetal loss, are recommended to undergo the invasive procedures. The risk stratification strategies that have been in current use include the assessment of maternal age, maternal serum biochemical markers and fetal ultrasound features (Nicolaides, K. H. et al., *Prenat Diagn* **22**:308-315 (2002)).

5 [0005] To attain better sensitivity and specificity, various combinations of markers and approaches have been evaluated (Wald, N. J. et al., *Prenat Diagn* **17**:821-829 (1997)), including the triple test, quadruple test (Wald, N. J. et al., *Lancet* **361**:835-836 (2003)), integrated test (Wald, N. J. et al., *N Engl J Med* **341**:461-467 (1999)) and first trimester screening (Wapner, R. et al., *N Engl J Med* **349**:1405-1413 (2003)). Serum biochemical
10 markers in use include, alpha-fetoprotein, unconjugated estriol, total or free beta-human chorionic gonadotropin, inhibin-A, and pregnancy-associated plasma protein-A (PAPP-A). Pregnancies whose risk is shown to be high by these screening modalities are ultimately referred for amniocentesis or CVS.

[0006] Recently, the discovery of circulating cell-free fetal nucleic acids in maternal
15 plasma has provided an alternative source of fetal genetic material which can be sampled noninvasively (Lo, Y. M. D. et al., *Lancet* **350**:485-487 (1997); Poon, L. L. M. et al., *Clin Chem* **46**:1832-1834 (2000)). Furthermore, the concentrations of circulating fetal DNA in the plasma of women carrying trisomy 21 fetuses have been shown to be significantly higher than those in women carrying euploid fetuses (Lo, Y. M. D. et al., *Clin Chem* **45**:1747-1751
20 (1999); Zhong, X. Y. et al., *Prenat Diagn* **20**:795-798 (2000)). Recently, circulating fetal RNA has also been shown to be promising as a class of gender-independent fetal nucleic acid markers in maternal plasma. (Ng, E. K. O. et al., *Clin Chem* **49**:727-731 (2003); Ng, E. K. O. et al., *Proc Natl Acad Sci U S A* **100**:4748-4753 (2003)). Thus, circulating fetal nucleic acid quantification is useful as an additional prenatal screening marker for the risk stratification of
25 pregnancies.

[0007] Placenta-expressed mRNA transcripts, such as those coding for *human placental lactogen (hPL)*, *human chorionic gonadotropin beta subunit (β hCG)* (Ng, E. K. O. et al., *Clin Chem* **49**:727-731 (2003)), *corticotropin releasing hormone (CRH)* (Ng, E. K. O. et al., *Proc Natl Acad Sci U S A* **100**:4748-4753 (2003b)), *tissue factor pathway inhibitor 2 (TFPI2)*,
30 *KISS-1 metastasis-suppressor (KISS1)* and *placenta-specific 1 (PLAC1)* (Tsui, N. B. Y. et al., *J Med Genet* **41**:461-7 (2004)), have been shown to be detectable in maternal plasma. These placenta-derived mRNA species are known to be pregnancy-specific (Ng, E. K. O. et al.,

Proc Natl Acad Sci U S A **100**:4748-4753 (2003); Tsui, N. B. Y. et al., *J Med Genet* **41**:461-7 (2004)). In particular, aberrant elevations in *CRH* mRNA concentrations in maternal plasma have been reported in preeclamptic pregnancies (Ng, E. K. O. et al., *Clin Chem* **49**:727-731 (2003)). As these placenta-expressed markers are pregnancy-specific but gender- and polymorphism-independent, they are useful in the noninvasive prenatal assessment of all pregnancies.

[0008] Chromosomal aneuploidy alters the dosage of genes located on the aneuploid chromosome. The altered gene dosage can be reflected by a distorted allele ratio of the genes. The distorted allele ratio is in turn reflected by a distorted ratio of alleles of polymorphisms present on the RNA transcript of the genetic loci on the aneuploid chromosome. One example of such polymorphisms is single nucleotide polymorphism (SNP) in which the ratio of the SNP alleles may be distorted in the presence of aneuploidy of the chromosome concerned. Thus, a reference range for the RNA-SNP ratio is established for normal pregnancies and fetal trisomy 21 can be determined when a deviation from the reference ratio is observed. In comparison to conventional cytogenetic methods of analysis, the proposed technique does not require prior culturing of the fetal cells and therefore shortens the analytical time. In addition, maternal blood samples can be obtained non-invasively, thus minimizing potential harm to both the fetus and the mother.

BRIEF SUMMARY OF THE INVENTION

[0009] In one embodiment, the present invention provides an improved method for detecting the presence of a chromosomal disorder in the fetus of a pregnant woman using the ratio of alleles of RNA transcript molecules from the pregnant woman's fetus in comparison to the ratio found in pregnant women with a chromosomally normal fetus. The use of the ratio in this manner provides superior sensitivity in detecting fetal chromosomal disorders, especially when compared to merely quantifying the amount of a particular allele or the total concentration of a particular RNA transcript present.

[0010] The first step of the method involves determining the ratio of alleles of the RNA transcripts in the fetus of a pregnant woman. This is accomplished by obtaining an RNA-containing biological sample from the pregnant woman, wherein the RNA-containing biological sample contains fetal RNA. The alleles are then discriminated from RNA transcribed from at least one genetic locus from at least one chromosome of concern,

followed by determination of the ratio of the alleles of the RNA transcripts. The second step involves comparing the ratio from the pregnant woman to a standard control representing an average ratio of alleles from comparable biological samples obtained from pregnant women each carrying a chromosomally normal fetus, wherein an increase or decrease in the ratio from the standard control indicates an increased risk of having a fetus with a chromosomal disorder.

[0011] In some embodiments, the present invention provides a method in which the chromosomal disorder is a member selected from the group consisting of trisomy 21, trisomy 18 and trisomy 13. In other embodiments, the chromosomal disorder is trisomy 21. In another embodiment, the chromosomal disorder is trisomy 13. In a further embodiment, the chromosomal disorder is trisomy 18. In yet another embodiment, the chromosomal disorder involves the X chromosome or the Y chromosome.

[0012] In another embodiment, the present invention provides a method in which the biological sample from the step of obtaining an RNA-containing biological sample is a member selected from the group consisting of maternal blood, maternal plasma or serum, amniotic fluid, a chorionic villus sample, biopsy material from a preimplantation embryo, fetal nucleated cells or fetal cellular remnants isolated from maternal blood, maternal urine, maternal saliva, washings of the female reproductive tract and a sample obtained by celocentesis. In still another embodiment, the biological sample is maternal blood. In yet another embodiment, the biological sample is a chorionic villus sample. In a further embodiment, the biological sample contains cellular elements or cellular remnants in maternal blood.

[0013] In other embodiments, the present invention provides a method in which the fetal RNA from the step of obtaining an RNA-containing biological sample is derived from the placenta.

[0014] In another embodiment, the present invention provides a method where the step of discriminating the alleles of RNA involves reverse transcriptase polymerase chain reaction (RT-PCR).

[0015] In a further embodiment, the present invention provides a method where the step of discriminating the alleles of RNA and/or the step of determining the ratio of the different alleles is performed using a member selected from the group consisting of a primer extension reaction, mass spectrometry, hybridization using at least one probe, hybridization using at

least one fluorescently labeled probe, direct sequencing, cloning and sequencing, and electrophoresis.

[0016] In some embodiments, the present invention provides a method in which the alleles of the steps involving discriminating the alleles of RNA, determining the ratio of the different alleles, and comparing the ratio from the previous step to a standard control are differentiated by sequence variation. In another embodiment, the sequence variation is a single nucleotide polymorphism (SNP). In a further embodiment, the sequence variation is an insertion/deletion polymorphism. In still another embodiment, the sequence variation is a simple tandem repeat polymorphism.

[0017] In other embodiments, the present invention provides a method in which the RNA is transcribed from a member selected from the group consisting of chromosome 21, chromosome 18, chromosome 13, chromosome X and chromosome Y. In another embodiment, the RNA is transcribed from chromosome 21. In a further embodiment, the RNA is transcribed from chromosome 18. In still another embodiment, the RNA is transcribed from chromosome 13.

[0018] In another embodiment, the present invention provides a method in which the RNA is expressed in the placenta at a level which is two-fold or more than that of maternal blood. In some embodiments, the RNA is expressed in the placenta at a level which is five-fold or more than that of maternal blood. In other embodiments, the RNA is expressed in the placenta at a level which is ten-fold or more than that of maternal blood.

[0019] In a further embodiment, the present invention provides a method in which the RNA is mRNA. In another embodiment, the RNA is transcribed from at least one genetic locus selected from the group consisting of *collagen VI alpha 1 (COL6A1)*, *superoxide dismutase 1 (SOD1)*, *collagen VI alpha 2 (COL6A2)*, *mitochondrial ATP synthase O subunit (ATP5O)*, *BTG family, member 3 (BTG 3)*, *a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 1 (ADAMTS1)*, *beta-site APP-cleaving enzyme 2 (BACE2)*, *intersectin 1 (ITSN1)*, *amyloid beta (A4) precursor protein (APP)*, *ATP synthase, H⁺ transporting, mitochondrial F0 complex, subunit F6 (ATP5J)*, *Down syndrome critical region gene 5 (DSCR5)*, *placenta-specific 4 (PLAC4)*, *hypothetical protein BC005107 (LOC90625)*, *ribosomal protein L17 (RPL17)*, *serpin peptidase inhibitor clade B (ovalbumin) member 2 (SERPINB2)* and *collagen type IV alpha 2 (COL4A2)*. In yet another embodiment, the RNA is transcribed from a genetic locus which contains a single nucleotide polymorphism. In

other embodiments, the RNA is transcribed from at least one genetic locus selected from the group consisting of *collagen VI alpha 1* (*COL6A1*) and *collagen VI alpha 2* (*COL6A2*). In still other embodiments, the RNA transcribed from the genetic locus the *COL6A1* SNP is Arg⁸⁵⁰_{His} or Ser⁹³²_{Ser}. In yet another embodiment, the RNA transcribed from the genetic locus
5 the *COL6A2* SNP is Val⁷²⁸_{Val}.

[0020] In another embodiment, the present invention provides a method in which the RNA is transcribed from the genetic locus for *placenta-specific 4* (*PLAC4*). In yet another embodiment, the RNA is transcribed from any variant of the *PLAC4* gene such as AF269287, AK027868, AK092431, BC093685, BC101615, BC101617, L13197, NM_182832 and
10 LOC191585. In still yet another embodiment, the RNA transcribed from the genetic locus of the *PLAC4* gene contains a single nucleotide polymorphism, or an insertion-deletion polymorphism selected from the group consisting of rs3804026, rs4818219, rs9977003, rs7844, rs9015, rs13643, rs9305729, rs9305730, rs5019195, rs5019194, rs5844069, rs1049904, rs16998089, rs12482116, rs11909439, rs7278659, rs12106409, rs12106395,
15 rs12106401, rs12106434, rs2183584, rs3949725, rs8130833, rs10222145 and rs9981478, or other polymorphisms located within the *PLAC4* gene locus such as *PLAC4*-41471145 and *PLAC4*-41476236.

[0021] In some embodiments, the present invention provides a method wherein the woman is during the first trimester of gestation. In other embodiments, the woman is during the
20 second or third trimester of gestation.

[0022] In a further embodiment, the present invention provides a method wherein the comparison step shows an increased risk of the fetus having a chromosomal disorder if the ratio of the alleles of the RNA transcripts in the fetus of a pregnant woman is higher or lower by 1 standard deviation from the standard control. In another embodiment, the comparison
25 step shows an increased risk of the fetus having a chromosomal disorder if the ratio of the alleles of the RNA transcripts in the fetus of a pregnant woman is higher or lower by 2 standard deviations from the standard control. In some other embodiments, the comparison step shows an increased risk of the fetus having a chromosomal disorder if the ratio of the alleles of the RNA transcripts in the fetus of a pregnant woman is higher or lower by 3
30 standard deviations from the standard control.

[0023] In another embodiment, the present invention provides a kit for detecting the presence of a fetus with a chromosomal disorder in a pregnant woman. One component of

the kit is primers for amplifying the region of interest. Another component of the kit is a standard control representing an average ratio of alleles from comparable biological samples obtained from pregnant women each carrying a chromosomally normal fetus. In yet another embodiment, a third component of the kit comprises hybridization probes for discriminating between the different alleles of each RNA species.

In accordance with an aspect of the present invention there is provided a method for detecting the presence of a chromosomal disorder in the fetus of a pregnant woman, comprising the steps of: (a) discriminating alleles from fetal RNA transcribed from at least one genetic locus from at least one chromosome of concern in an RNA-containing biological sample from the pregnant woman, wherein the RNA-containing biological sample contains fetal RNA and said genetic locus is selected from the group consisting of *collagen V1 alpha 1* (COL6A1), *collagen V1 alpha 2* (COL6A2), *placenta-specific 4* (PLAC4), *serpin peptidase inhibitor clade B (ovalbumin) member 2* (SERPINB2) and *collagen type IV alpha 2* (COL4A2); (b) determining a ratio of the alleles of the RNA transcripts; and (c) comparing the ratio from step (b) to a standard control representing a ratio of alleles from comparable biological samples obtained from pregnant women each carrying a chromosomally normal fetus, wherein an increase or decrease in the determined ratio of step (b) to the standard control indicates an increased risk of having a fetus with a chromosomal disorder.

In accordance with a further aspect of the present invention there is provided a kit for detecting the presence of a fetus with a chromosomal disorder in a pregnant woman, the kit comprising: (a) primers for amplifying the alleles from fetal RNA transcribed from at least one genetic locus from at least one chromosome of concern in an RNA-containing biological sample said genetic locus is selected from the group consisting of *collagen V1 alpha 1* (COL6A1), *collagen V1 alpha 2* (COL6A2), *placenta-specific 4* (PLAC4), *serpin peptidase inhibitor clade B (ovalbumin) member 2* (SERPINB2) and *collagen type IV alpha 2* (COL4A2); and (b) a standard control of DNA or RNA representing a ratio of alleles from comparable biological samples obtained from pregnant women each carrying a chromosomally normal fetus.

In further aspects of the invention, the RNA is a variant transcribed from the *PLAC4* locus, selected from the group consisting of AF269287, AK027868, AK092431, BC093685, BC101615, BC101617, L13197, NM_182832 and LOC191585.

BRIEF DESCRIPTION OF THE DRAWINGS

[0024] Figure 1. Schematic illustration of one embodiment of the strategy employed for the noninvasive determination of fetal trisomy 21 through maternal blood, serum or plasma analysis by the relative quantification of alleles of placenta-expressed transcripts located on chromosome 21. (A) Normal and trisomy 21 fetuses heterozygous at a transcribed SNP locus on chromosome 21. The fetus with trisomy 21 has an extra copy of the gene. “A” and “G” denote the respective alleles of the transcribed SNP. (B) The gene is expressed in placental tissues and the resultant transcripts are allelic by exhibiting the coding SNP’s. The ratio of the two RNA alleles in trisomy 21 placenta is expected to deviate from that of the normal placenta, due to the expression of an extra copy of the gene. (C) The RNA transcripts are released into maternal blood and their relative abundance is reflective of the placental gene expression profile. Thus, the maternal blood, serum or plasma ratio of the two RNA alleles in a trisomy 21 pregnancy is expected to deviate from that of a normal pregnancy.

[0025] Figure 2. Box plots of concentrations of placenta-derived mRNA transcripts in maternal plasma of non-pregnant women, and women in the first and third trimesters of pregnancies, respectively. (A) *COL6A1* mRNA. (B) *SOD1* mRNA. (C) *COL6A2* mRNA and (D) *ATP50* mRNA. *Control*, non-pregnant women; *1st trimester*, women in the first trimester of pregnancy; *3rd trimester*, women in the third trimester of pregnancy. The lines inside the boxes denote the medians. The boxes mark the interval between the 25th and 75th percentiles. The error bars denote the interval between 10th and 90th percentiles. The filled circles mark the data points outside the 10th and 90th percentiles.

[0026] Figure 3. Clearance of placenta-derived mRNA transcripts from maternal plasma after delivery. (A) *COL6A1*, (B) *SOD1*, (C) *COL6A2* and (D) *ATPSO* mRNA concentrations in maternal plasma before and at 24 hours after delivery. Each line represents a paired plasma sample obtained from one subject.

[0027] **Figure 4.** Ratios between the two transcript alleles for the SNP rs1053312 of *COL6A1* gene in placental tissues. The relative quantities between the RNA alleles were determined by allele-specific real-time quantitative RT-PCR. RNA from normal CVS (*CVS*), normal term placentas (*PLN*) and trisomy 21 placentas (*T21*) were assayed. The ratios were
5 calculated by the difference in threshold cycle values (ΔCt).

[0028] **Figure 5.** Ratios between the two transcript alleles for the SNP rs2839114 of *COL6A2* gene in placental tissues as determined by allele-specific real-time quantitative RT-PCR. RNA from normal CVS (*CVS*), normal term placentas (*PLN*) and trisomy 21 placentas (*T21*) were assayed. The ratios were calculated either by (A) the difference in threshold cycle
10 values (ΔCt) or (B) the difference in their fluorescent intensities attained in the last PCR cycle ($\Delta \Delta Rn$).

[0029] **Figure 6.** Ratios of allelic frequencies of SNP rs1053320 (*COL6A1*) in placentas of heterozygous normal and trisomy 21 fetuses determined by primer extension followed by mass spectrometry analysis. (A) Placental DNA. (B) Placental RNA.

15 [0030] **Figure 7.** Ratios of allelic frequencies of SNP rs2839114 (*COL6A2*) in placentas of heterozygous normal and trisomy 21 fetuses determined by primer extension followed by mass spectrometry analysis. (A) Placental DNA. (B) Placental RNA.

[0031] **Figure 8.** *PLAC4* transcript allelic ratios in placentas of pregnant women carrying normal and trisomy 21 fetuses. The allelic ratios were determined by primer extension
20 followed by mass spectrometry analysis using SNP rs8130833.

[0032] **Figure 9.** *PLAC4* transcript allelic ratios in the plasma samples of pregnant women carrying normal and trisomy 21 fetuses.

[0033] **Figure 10.** Correlation of allelic ratios of the *PLAC4* transcript from placentas and maternal plasma samples.

25 [0034] **Figure 11.** Comparison of maternal plasma *PLAC4* mRNA concentration in trisomy 21 and control pregnancies. The boxes mark the interval between the 25th and 75th percentiles. The whiskers denote the interval between the 10th and 90th percentiles. The filled circles mark the data points outside the 10th and 90th percentiles.

[0035] **Figure 12.** *SERPINB2* transcript allelic ratios in placentas of pregnant women carrying normal and trisomy 18 fetuses. The allelic ratios were determined by primer extension followed by mass spectrometry analysis using SNP rs6098.

[0036] **Figure 13.** *COL4A2* transcript allelic ratios in placentas of pregnant women carrying normal and trisomy 13 fetuses. The allelic ratios were determined by primer extension followed by mass spectrometry analysis using SNP rs7990383.

DETAILED DESCRIPTION OF THE INVENTION

I. Definitions

[0037] As used herein, the term “chromosomal disorder” usually refers to a state of chromosomal abnormality where the number of chromosomes is not an exact multiple of the usual haploid number: frequently, there is either an additional chromosome or one missing. In some cases, “chromosomal disorder” may also refer to a state of chromosomal abnormality where a proportion of one or more chromosomes is not an exact multiple of the usual haploid number, due to, for example, chromosome translocation. A common chromosomal disorder is aneuploidy. A common form of chromosomal aneuploidy is a trisomy, where a single additional chromosome is present. For example, trisomy 18 is a chromosomal abnormality where a third chromosome 18 is found in a cell, whereas a third chromosome 21 is present in the cells of a patient suffering from trisomy 21. Chromosomal translocation (e.g. translocation between chromosome 21 and 14 where some of the 14th chromosome is replaced by extra 21st chromosome) may cause partial trisomy 21.

[0038] As used herein, the term “RNA-containing biological sample” refers to a biological sample (such as those discussed below) that contains ribonucleic acid (RNA). RNA refers to a polymer of ribonucleotides that has a sequence corresponding to at least a portion of a pre-selected location in the human genome. RNA as used herein includes, but is not limited to, mRNA, ribosomal RNA and micro RNA. RNA can be protein encoding sequences such as mRNA, or non-coding sequences such as ribosomal RNA, microRNA or other transcribed sequences without well-defined functions. mRNA is an RNA molecule transcribed from the DNA of a gene, and from which a protein is translated by the action of ribosomes. Ribosomal RNA (rRNA) is a non-coding RNA that is not translated into a protein. Micro RNA (miRNA) is a sub-type of “small RNAs” that are distinguished by their origins, not their functions. Micro RNA is less than 30 nucleotides in length, and is transcribed from

DNA but not translated into protein. One of skill in the art will appreciate that other types of RNA are useful in the present invention.

[0039] As used herein, the terms “fetal”, “placental derived” and “placental expressed” refer to the origin of certain RNA species that are detectable in a biological sample from a pregnant woman, e.g., blood. For example, a fetal RNA species is one that has been transcribed from a fetal DNA sequence. Placental-derived or placental expressed RNA is one type of fetal RNA. One of skill in the art will appreciate that other fetal RNA are useful in the present invention. A placental derived or placental expressed RNA species is one that is transcribed in the placenta.

[0040] As used herein, the term “discriminating alleles from RNA transcribed from at least one genetic locus from at least one chromosome of concern” refers to the detection and quantification of particular RNA alleles transcribed from a particular genetic locus on a chromosome. The detection and quantification of alleles can be carried out by a variety of methods, including the use of hybridization probes and quantitative real time polymerase chain reaction (QRT-PCR). Other methods include the use of mass spectrometry (MS), electrophoresis, pyrosequencing, primer extension microarrays, chips and sequencing.

[0041] As used herein, the term “ratio of the alleles” refers to the ratio of the population of one allele and the population of the other allele in a biological sample. In some cases, it is possible that in trisomies a fetus may be tri-allelic for a particular locus. In such cases, the term “ratio of the alleles” refers to the ratio of the population of any one allele against one of the other alleles, or any one allele against the other two alleles.

[0042] As used herein, the term “standard control” refers to a sample suitable for the use of a method of the present invention, in order for determining the ratio of the RNA-SNP alleles transcribed from a particular genetic locus, e.g., *COL6A1*, *SOD1*, *COL6A2*, *ATP5O*, *BTG3*, *ADAMTS1*, *BACE2*, *ITSN1*, *APP*, *ATP5J*, *DSCR5*, *PLAC4*, *LOC90625*, *RPL17*, *SERPINB2* or *COL4A2*. Such sample contains a known ratio of the RNA-SNP alleles transcribed from a particular genetic locus that closely reflects the average ratio of such RNA-SNP alleles in pregnant women who each carries a chromosomally normal fetus. The standard control can also represent the mean ratio, the median ratio, or another useful ratio known to one of skill in the art. Determination of the standard control is described in greater detail below.

[0043] As used herein, the term “pregnant women” refers to a group of pregnant women who each carries a chromosomally normal fetus, and refers to certain characteristics, such as

the ratio of RNA alleles transcribed from the loci of interest, that is representative of a randomly selected group of women who carry chromosomally normal fetuses. This selected group should comprise a sufficient number of women such that the average, mean, median or other mathematical relationship, ratio of RNA alleles transcribed from the loci of interest
5 among these women reflects, with reasonable accuracy, the ratio of RNA alleles in the general population of healthy pregnant women with healthy fetuses. The mother can be screened for the risk of fetal chromosomal aneuploidy during the first trimester of pregnancy, about the first 13 weeks of gestation. The mother can also be screened during the second or third trimester of gestation. The second trimester of gestation is from about 14 to about 27
10 weeks of gestation. The third trimester of gestation is from about 28 weeks to the end of gestation, about 40 weeks. Moreover, the preferred gestational age for testing may also depend on the RNA marker used in testing.

[0044] As used herein, the term “chromosomally normal” refers to the state where the number of chromosomes is an exact multiple of the haploid number, such as twice the
15 number of chromosomes found in a haploid, and each chromosome is present in the same number (except the sex chromosomes in the case of, e.g., male humans, where two different sex chromosomes, X and Y, are present at one copy each).

[0045] As used herein, the term “an increase or a decrease in the ratio from the standard control” refers to a positive or negative change in the ratio as compared to the standard
20 control. An increase is preferably at least 10%, more preferably at least 50%, and most preferably at least 100%. Similarly, a decrease is preferably at least 10%, more preferably at least 50%, and most preferably at least 90%. In addition, the increase or decrease in the ratio can be at least 1 standard deviation from the standard control. The increase or decrease can also be at least 2 standard deviations from the standard control. The increase or decrease can
25 also be at least 3 standard deviations from the standard control.

[0046] As used herein, the term “single nucleotide polymorphism” (SNP) refers to a nucleic acid sequence variation involving the alteration of a single nucleotide. SNPs useful in the present invention include those present on the corresponding RNA transcripts transcribed from the genetic loci of interest including, but not limited to, *collagen VI alpha 1*
30 (*COL6A1*), *superoxide dismutase 1* (*SOD1*), *collagen VI alpha 2* (*COL6A2*), *mitochondrial ATP synthase O subunit* (*ATP5O*), *BTG family, member 3* (*BTG 3*), *a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 1* (*ADAMTS1*), *beta-site*

APP-cleaving enzyme 2 (BACE2), intersectin 1 (ITSN1), amyloid beta (A4) precursor protein (APP), ATP synthase, H⁺ transporting, mitochondrial F0 complex, subunit F6 (ATP5J), Down syndrome critical region gene 5 (DSCR5), placenta-specific 4 (PLAC4), hypothetical protein BC005107 (LOC90625), ribosomal protein L17 (RPL17), serpin peptidase inhibitor clade B (ovalbumin) member 2 (SERPINB2) and collagen type IV alpha 2 (COL4A2). Such SNPs are termed RNA-SNPs since they involve the alteration of a single nucleotide on RNA. Alleles of the RNA-SNPs are then used in the present invention to determine a ratio of the RNA-SNP alleles in a biological sample obtained from a pregnant woman and to compare that ratio to the ratio obtained from biological samples obtained from a group of pregnant women each with a normal fetus. One of skill in the art will appreciate that other markers and insertion/deletion polymorphisms are also useful in the present invention.

[0047] As used herein, the term “maternal blood” refers to a blood sample or preparation from a pregnant woman or a woman being tested for possible pregnancy. The term encompasses whole blood or any fractions of blood. Examples of “maternal blood” include plasma and serum. A maternal blood sample that is essentially free of cells is also referred to as “acellular,” where generally no platelets are present.

[0048] As used herein, the term “cellular elements or cellular remnants” refers to parts of cells that remain in the biological sample, including, but not limited to, platelets, apoptotic bodies and syncytiotrophoblast microparticles.

II. Methods of the present invention

[0049] The present invention uses the pregnancy-specificity of fetal-expressed transcripts to develop a method which allows the genetic determination of fetal chromosomal aneuploidy and thus the establishment of its diagnosis noninvasively. In one embodiment, the fetal-expressed transcripts are those expressed in the placenta. Specifically, the present invention detects single nucleotide polymorphisms (SNPs) from RNA transcripts with tissue-specific expression patterns that are encoded by genes on the aneuploid chromosome. Other polymorphisms are also detectable by the methods of the present invention, such as an insertion/deletion polymorphism and a simple tandem repeat polymorphism. The status of the locus is determined through the assessment of the ratio between informative SNPs on the RNA transcribed from the genetic loci of interest. In short, the present invention compares the ratio between alleles of a polymorphic site on locus- and tissue-specific RNA transcripts from an aneuploid fetus to a euploid fetus.

[0050] The present invention, therefore, can be applied to the prenatal diagnosis of trisomy 21 which involves the analysis of informative SNPs on RNA transcripts with placental tissue expression that are derived from loci on chromosome 21. Fetal trisomy 21 is then determined by comparing the ratios between the informative SNPs through the detection of the placenta-expressed RNA transcripts in maternal blood. The fetal-specificity of the markers in maternal blood is conferred by their placental tissue expression, while the aneuploid status is determined by the abnormal ratios between the informative SNPs on the RNA transcripts.

[0051] The methods of the present invention enable the genetic determination of fetal trisomy 21 and its prenatal diagnosis based on the analysis of placenta-expressed RNA-SNPs encoded by genetic loci on chromosome 21. The RNA-SNP alleles can be discriminated by, for example, real-time QRT-PCR assays and the ratio between them, which is indicative of the gene dosage of the locus, is determined. In another embodiment, the RNA-SNP alleles can be discriminated by primer extension followed by mass spectrometry.

A. Chromosomal disorders detectable using the methods of the present invention

[0052] The present invention provides methods for the detection of chromosomal aneuploidies such as trisomy 21. The methods also enable detection of other fetal aneuploidies, such as those present on, or that involve, chromosomes 18, 13, X and Y. The methods also enable the noninvasive detection of fetal chromosomal aneuploidy when maternal blood is analyzed. The utility of RNA-SNP detection does extend beyond the detection of chromosomal abnormalities to the detection of other genetic variations of the fetus, e.g. paternally-inherited polymorphisms and mutations.

B. Biological samples useful in the present invention for detecting fetal chromosomal disorders

[0053] The first step of practicing the present invention is to obtain a biological sample from a pregnant woman at a gestational age suitable for testing using a method of the present invention, or from a woman who is being tested for possible pregnancy. The suitable gestational age may vary depending on the disorder tested and sometimes the RNA marker used, as discussed above.

[0054] The biological sample can be maternal blood, including maternal plasma or serum. In some circumstances, the biological sample is acellular. In other circumstances, the

biological sample does contain cellular elements or cellular remnants in maternal blood. Other biological samples include amniotic fluid, chorionic villus sample, biopsy material from a preimplantation embryo, maternal urine, maternal saliva, a celocentesis sample, fetal nucleated cells or fetal cellular remnants, or the sample obtained from washings of the female reproductive tract.

[0055] When the biological sample is blood, collection of blood from a woman is performed in accordance with the standard protocol hospitals or clinics generally follow. An appropriate amount of peripheral blood, e.g., between 3-20 ml, is collected and can be stored according to standard procedure prior to further preparation.

10 [0056] Biological samples useful in the present invention contain fetal RNA. The fetal RNA can be transcribed from chromosomes 21, 18, 13, X and Y. In addition, the level at which RNA is expressed in the placenta can be two times, five times or ten times or more the level expressed in the maternal blood or its various fractions.

15 [0057] The fetal RNA is typically derived from any tissue of fetal origin including, but not limited to, the placenta, and can be mRNA. The RNA can be extracted from the biological sample by a variety of methods. The general methods of RNA preparation (e.g., described by Sambrook and Russell, *Molecular Cloning: A Laboratory Manual* 3d ed., 2001) can be followed; various commercially available reagents or kits, such as Trizol reagent (Invitrogen, Carlsbad, CA), Oligotex Direct mRNA Kits (Qiagen, Valencia, CA), RNeasy Mini Kits
20 (Qiagen, Hilden, Germany), and PolyATtract® Series 9600™ (Promega, Madison, WI), may also be used to obtain RNA from a blood sample from a woman. Combinations of more than one of these methods may also be used. Careful handling of the samples, thorough treatment with DNase, and proper negative controls in the amplification and quantification steps should be used to eliminate DNA from the RNA preparations.

25 C. Methods for discriminating alleles of RNA transcripts

[0058] Discriminating the alleles of RNA transcripts can be accomplished by a variety of methods, including PCR, mass spectrometry (MS), gel electrophoresis, pyrosequencing, primer extension assays, chips, sequencing and hybridization with one or more fluorescent probes.

1. PCR-based discrimination of RNA-SNP alleles

[0059] Once RNA is extracted from the biological sample, the amount of each particular SNP allele of the RNA of interest, e.g., *COL6A1*, *SOD1*, *COL6A2*, *ATP5O*, *BTG3*, *ADAMTS1*, *BACE2*, *ITSN1*, *APP*, *ATP5J*, *DSCR5*, *PLAC4*, *LOC90625*, *RPL17*, *SERPINB2* or *COL4A2*, may be assessed.

[0060] Variants of the selected gene are also useful in the present invention. For example, useful variants of the *PLAC4* gene include variants identified by the GenBank accession numbers: AF269287, AK027868, AK092431, BC093685, BC101615, BC101617, L13197, NM_182832 and LOC191585. The RNA transcribed from the genetic locus of the *PLAC4* gene contains one or more single nucleotide polymorphism, or insertion-deletion polymorphism. Exemplary polymorphisms transcribed from the *PLAC4* gene include, but are not limited to, polymorphisms identified by the database of SNPs (a website having a database of these gene sequences and nucleic acid sequences is the NCBI Database Search Engine offered at the time of preparation of this application based in the U.S.) accession numbers (with *PLAC4* coordinates based on the Human May 2004 (hg17) assembly at the UCSC Genome Browser (a website having a database of these gene sequences and nucleic acid sequences is the UCSC Genome Browser developed and maintained by the Genome Bioinformatics Group offered at the time of preparation of this application based in the U.S.): rs3804026 (*PLAC4*-41469163), rs4818219 (*PLAC4*-41469764), rs9977003 (*PLAC4*41470591), rs7844 (*PLAC4*-41470699), rs9015 (*PLAC4*-41470877), rs13643 (*PLAC4*41471296), rs9305729 (*PLAC4*-41472272), rs9305730 (*PLAC4*-41472277), rs5019195 (*PLAC4*-41473295), rs5019194 (*PLAC4*-41473302), rs5844069 (*PLAC4*-41473306), rs1049904 (*PLAC4*-41473392), rs16998089 (*PLAC4*-41473496), rs12482116 (*PLAC4*-41475590), rs11909439 (*PLAC4*-41475912), rs7278659 (*PLAC4*-41476875), rs12106409 (*PLAC4*-41477273), rs12106395 (*PLAC4*-41477340), rs12106401 (*PLAC4*-41477425), rs12106434 (*PLAC4*-41477486), rs2183584 (*PLAC4*-41477956), rs3949725 (*PLAC4*41478283), rs8130833 (*PLAC4*-41478755), rs10222145 (*PLAC4*-41480512) and rs9981478 (*PLAC4*-41480564), or other polymorphisms located within the *PLAC4* gene locus such as *PLAC4*-41471145 and *PLAC4*-41476236, which are located at the nucleotide coordinates of 41471145 and 41476236, respectively, on chromosome 21 based on the Human May 2004 (hg17) assembly at the UCSC Genome Browser (a website having a database of these gene sequences and nucleic acid sequences for genomes offered at the time of preparation of this application based in the U.S.). Those of skill in the art will appreciate that other polymorphisms in *PLAC4* are also useful in the present invention.

[0061] The dbSNP accession numbers refer only to the SNP portion of the genomic sequence on which the SNP is located. The extent of the sequence provided for the SNP can be selected for both the upstream and downstream portions of the transcribed genomic sequence.

[0062] Other RNA-SNPs useful in the present invention include ^{Arg850}_{His} of *COL6A1* (*COL6A1*-46247817), ^{Ser932}_{Ser} of *COL6A1* (*COL6A1*-46248064) and ^{Val728}_{Val} of *COL6A2* (*COL6A2*-46370341), on chromosome 21 based on the Human May 2004 (hg17) assembly at the UCSC Genome Browser (a website having a database of these gene sequences and nucleic acid sequences for genomes offered at the time of preparation of this application based in the U.S.).

[0063] In most instances, it is desirable to amplify the target sequence using any of several nucleic acid amplification procedures which are well known in the art (listed above and described in greater detail below). Specifically, nucleic acid amplification is the enzymatic synthesis of nucleic acid amplicons (copies) which contain a sequence that is complementary to a nucleic acid sequence being amplified. Nucleic acid amplification is especially beneficial when the amount of target sequence present in a sample is very low. By amplifying the target sequences and detecting the amplicon synthesized, the sensitivity of an assay can be vastly improved, since fewer target sequences are needed at the beginning of the assay to better ensure detection of nucleic acid in the sample belonging to the organism or virus of interest.

[0064] Amplification of polynucleotides utilizes methods such as the polymerase chain reaction (PCR), ligation amplification (or ligase chain reaction (LCR)) and amplification methods based on the use of Q-beta replicase. Also useful are strand displacement amplification (SDA), thermophilic SDA, nucleic acid sequence based amplification (3SR or NASBA) and transcription-associated amplification (TAA). These methods are well known and widely practiced in the art. Reagents and hardware for conducting PCR are commercially available.

[0065] Prior to the amplification step, it is typically necessary to synthesize a DNA copy (cDNA) of the RNA transcript of interest. This is achieved by reverse transcription, which can be carried out as a separate step, or in a homogeneous reverse transcription-polymerase chain reaction (RT-PCR), a modification of the polymerase chain reaction for amplifying RNA. Methods suitable for PCR amplification of ribonucleic acids are described by Romero and Rotbart in *Diagnostic Molecular Biology: Principles and Applications* pp.401-406; Persing et al., eds., Mayo Foundation, Rochester, MN, 1993; Egger et al., *J. Clin. Microbiol.* 33:1442-1447, 1995; and U.S. Patent No. 5,075,212.

[0066] The general methods of PCR are well known in the art and are thus not described in detail herein. For a review of PCR methods, protocols, and principles in designing primers, see, e.g., Innis, et al., *PCR Protocols: A Guide to Methods and Applications*, Academic Press,

Inc. N.Y., 1990. PCR reagents and protocols are also available from commercial vendors, such as Roche Molecular Systems.

5 [0067] PCR is usually carried out as an automated process with a thermostable enzyme. In this process, the temperature of the reaction mixture is cycled through a denaturing region, a primer annealing region, and an extension reaction region automatically. Machines specifically adapted for this purpose are commercially available.

[0068] Primers useful to amplify the RNA transcript sequences are preferably complementary to, and hybridize specifically to sequences that flank a target region therein. The polynucleotide sequences generated by amplification may be sequenced directly.
10 Alternatively, the amplified sequence(s) may be cloned prior to sequence analysis. As a third possibility, primers may be designed to overlap the SNP site for the practice of allele-specific PCR. Allele-specific PCR enables the discrimination of RNA-SNP alleles as only the correctly hybridized primers will be amplified. PCR primers refers to oligonucleotides that can be used in a polymerase chain reaction (PCR) to amplify a nucleotide sequence
15 originated from an RNA species transcribed from a locus of interest, such as *COL6A1*, *SOD1*, *COL6A2*, *ATP5O*, *BTG3*, *ADAMTS1*, *BACE2*, *ITSN1*, *APP*, *ATP5J*, *DSCR5*, *PLAC4*, *LOC90625*, *RPL17*, *SERPINB2* or *COL4A2*. At least one of the PCR primers for amplification of a nucleotide sequence encoding an above-named transcript should be sequence-specific for the locus.

20 [0069] Although PCR amplification of the target RNA-SNP alleles is typically used in practicing the present invention, one of skill in the art will recognize that amplification of the RNA species in a maternal blood sample may be accomplished by any known method, such as ligase chain reaction (LCR), transcription-mediated amplification, and self-sustained sequence replication or nucleic acid sequence-based amplification (NASBA), each of which
25 provides sufficient amplification. More recently developed branched-DNA technology may also be used to amplify the signal of RNA markers in maternal blood. For a review of branched-DNA (bDNA) signal amplification for direct quantification of nucleic acid sequences in clinical samples, see Nolte, *Adv. Clin. Chem.* 33:201-235, 1998.

2. Other methods for the discrimination of RNA-SNP alleles

30 [0070] The RNA-SNP alleles of interest can also be detected using other standard techniques, well known to those of skill in the art. Although the detection step is typically

preceded by an amplification step, amplification is not required in the methods of the invention. For instance, the RNA transcript may be identified by size fractionation (e.g., gel electrophoresis), whether or not preceded by an amplification step. After running a sample in an agarose or polyacrylamide gel and labeling with ethidium bromide according to well known techniques (see, Sambrook and Russell, *Molecular Cloning: A Laboratory Manual* 3d ed., 2001), the presence of a band of the same size as the standard control is an indication of the presence of a target RNA sequence, the amount of which may then be compared to the control based on the intensity of the band. Alternatively, oligonucleotide probes specific to RNA encoding, e.g., *COL6A1*, *SOD1*, *COL6A2*, *ATP5O*, *BTG3*, *ADAMTS1*, *BACE2*, *ITSN1*, *APP*, *ATP5J*, *DSCR5*, *PLAC4*, *LOC90625*, *RPL17*, *SERPINB2* or *COL4A2*, can be used to detect the presence of such RNA species and indicate the amount of RNA molecules in comparison to the standard control, based on the intensity of signal imparted by the probe.

[0071] Sequence-specific probe hybridization is a well known method of detecting a particular nucleic acid comprising other species of nucleic acids. Under sufficiently stringent hybridization conditions, the probes hybridize specifically only to substantially complementary sequences. The stringency of the hybridization conditions can be relaxed to tolerate varying amounts of sequence mismatch.

[0072] A number of hybridization formats well known in the art, including but not limited to, solution phase, solid phase, or mixed phase hybridization assays. The following articles provide an overview of the various hybridization assay formats: Singer et al., *Biotechniques* 4:230, 1986; Haase et al., *Methods in Virology*, pp. 189-226, 1984; Wilkinson, *In situ Hybridization*, Wilkinson ed., IRL Press, Oxford University Press, Oxford; and Hames and Higgins eds., *Nucleic Acid Hybridization: A Practical Approach*, IRL Press, 1987.

[0073] The hybridization complexes are detected according to well known techniques and the detection is not a critical aspect of the present invention. Nucleic acid probes capable of specifically hybridizing to a target nucleic acid, i.e., the mRNA or the amplified DNA, can be labeled by any one of several methods typically used to detect the presence of hybridized nucleic acids. One common method of detection is the use of autoradiography using probes labeled with ^3H , ^{125}I , ^{35}S , ^{14}C , or ^{32}P , or the like. The choice of radioactive isotope depends on research preferences due to ease of synthesis, stability, and half lives of the selected isotopes. Other labels include compounds (e.g., biotin and digoxigenin), which bind to antiligands or antibodies labeled with fluorophores, chemiluminescent agents, and enzymes.

Alternatively, probes can be conjugated directly with labels such as fluorophores, chemiluminescent agents or enzymes. The choice of label depends on sensitivity required, ease of conjugation with the probe, stability requirements, and available instrumentation.

5 [0074] The probes and primers necessary for practicing the present invention can be synthesized and labeled using well known techniques. Oligonucleotides used as probes and primers may be chemically synthesized according to the solid phase phosphoramidite triester method first described by Beaucage and Caruthers, *Tetrahedron Letts.*, **22**:1859-1862, 1981, using an automated synthesizer, as described in Needham-VanDevanter et al., *Nucleic Acids Res.* **12**:6159-6168, 1984. Purification of oligonucleotides is by either native acrylamide gel electrophoresis or by anion-exchange high-performance liquid chromatography (HPLC) as described in Pearson and Regnier, *J. Chrom.*, **255**:137-149, 1983.

15 [0075] Other useful methods for discriminating alleles of RNA transcripts include direct sequencing, such as pyrosequencing. The process of pyrosequencing involves a primer-template complex where each of the four deoxynucleotide triphosphates is added one at a time. When the deoxynucleotide triphosphate is incorporated by the DNA polymerase, light is emitted. The amount of light generated is proportional to the number of bases added. Accordingly, the downstream sequence can be inferred.

20 [0076] The primer extension reaction is also useful in the present invention. The primer extension reaction operates by discriminating the SNP alleles by the incorporation of deoxynucleotides and/or dideoxynucleotides to a primer extension primer which hybridizes to a region adjacent to the SNP site. The primer is extended with a polymerase. The primer extended SNP can be detected physically by mass spectrometry or by a tagging moiety such as biotin. As the SNP site is only extended by a complementary deoxynucleotide or dideoxynucleotide that is either tagged by a specific label or generates a primer extension product with a specific mass, the SNP alleles can be discriminated.

25 [0077] Mass spectrometry methods enable the detection of a polynucleotide, for example a PCR amplicon or a primer extension product. The presence of the polynucleotide sequence is verified by comparing the mass of the detected signal with the expected mass of the polynucleotide of interest. The relative signal strength for a particular polynucleotide sequence indicates the relative population of a specific allele, thus enabling calculation of the allele ratio directly from the data. For a review of genotyping methods by mass spectrometry, see Pusch et al., *Pharmacogenomics* **3**:537-548, 2002.

D. Determining the ratio of alleles of RNA-SNPs

[0078] Generally, the determination of the ratio of the alleles of RNA-SNPs involves calculating the relative population of each RNA-SNP allele that is present in the sample, and dividing the value determined for one RNA-SNP allele by the value for the other RNA-SNP allele. Using a PCR based detection system entails dividing the label intensity associated with the PCR, primer extension or hybridization reaction products generated from one of the alleles with that of the other allele of the RNA transcript. Other methods for determining the ratio of alleles of RNA-SNP include comparing the abundance of sequencing products or the number of cloned sequences for each of the alleles. The RNA-SNP ratio can also be determined by comparing the mass signal intensities of the alleles by mass spectrometry.

[0079] Alternatively, the RNA-SNP ratio can be determined from the difference in the fluorescent intensity (ΔR_n) accumulated for each allele during a reaction, or from the number of PCR cycles required for a reaction of each allele to have accumulated a threshold fluorescent intensity (C_t). The ΔC_t and $\Delta \Delta R_n$ are reflective of the RNA-SNP allele ratio because these values are proportional to the logarithm of the amount of the RNA allele. As a result, the difference in the threshold cycle values (ΔC_t) or the difference in the accumulated fluorescent intensities ($\Delta \Delta R_n$) for each allele is reflective of the SNP ratio for a RNA transcript. For example, allele A can be detected with a FAM (6-carboxyfluorescein) labeled probe and allele B can be detected with a fluorescent probe such as VIC (from Applied Biosystems). The C_t and the ΔR_n values are calculated for each of allele A and allele B. The difference of the two C_t values is determined using threshold cycle values, and affords the ΔC_t value. The difference in the two ΔR_n values is determined using the accumulated fluorescent intensities and affords the $\Delta \Delta R_n$ value. As the C_t and ΔR_n values are logarithmically related to the abundance of PCR products, the difference in the C_t and ΔR_n values for each RNA-SNP is calculated and that difference (the ΔC_t and $\Delta \Delta R_n$ values) is reflective of the RNA-SNP ratio between the two RNA alleles.

E. Comparison of the RNA-SNP ratio to a standard control

[0080] Once the ratio of alleles has been determined in the subject, the ratio is compared to the standard control in order to determine the presence of fetal aneuploidy. A RNA-SNP ratio that is either higher or lower when compared to a known value determined in the control sample, indicates the presence of a fetal aneuploidy. For example, a ΔC_t value, $\Delta \Delta R_n$ value

or the label or mass intensity ratios between the SNP alleles that is either higher or lower when compared to a known value determined in the control sample, indicates the presence of a fetal aneuploidy.

[0081] In order to establish a standard control, a group of healthy pregnant women carrying healthy fetuses are first selected. These women are preferably, but not necessarily, of similar gestational age, which is within the appropriate time period of pregnancy for screening of conditions such as fetal chromosomal aneuploidy using the methods of the present invention. Similarly, a standard control is established using samples from a group of healthy non-pregnant women. The healthy status of the selected pregnant women and the fetuses they are carrying is confirmed by well established, routinely employed methods including, but not limited to, cytogenetic analysis or conducting fetal genetic analysis using the methods described above for obtaining a biological sample. The standard control can be determined prior to testing for the presence of fetal aneuploidy.

[0082] Furthermore, the selected group of healthy pregnant women carrying healthy fetuses must be of a reasonable size, such that the average, mean or median ratio of RNA-SNP alleles encoding *COL6A1*, *SOD1*, *COL6A2*, *ATP5O*, *BTG3*, *ADAMTS1*, *BACE2*, *ITSN1*, *APP*, *ATP5J*, *DSCR5*, *PLAC4*, *LOC90625*, *RPL17*, *SERPINB2* or *COL4A2*, calculated from the group can be reasonably regarded as representative of the normal or average, mean or median amount among the general population of healthy women carrying healthy fetuses. In some instances, the group comprises at least 10 women.

[0083] Once an average value is established for the ratio of RNA-SNP alleles based on the individual values found in each women of the selected group, this value is considered a standard for the RNA species. Any blood sample that contains a similar ratio of RNA of the same species can thus be used as a standard control. A solution containing RNA encoding *COL6A1*, *SOD1*, *COL6A2*, *ATP5O*, *BTG3*, *ADAMTS1*, *BACE2*, *ITSN1*, *APP*, *ATP5J*, *DSCR5*, *PLAC4*, *LOC90625*, *RPL17*, *SERPINB2* or *COL4A2*, with a ratio of the established average of the same species can also be artificially assembled and serve as a standard control.

[0084] An increase in the RNA-SNP ratio that is at least 10% higher as compared to the average value in the control sample, indicates an increased risk of having a fetus with chromosomal disorder. In some cases, the increase in the RNA-SNP ratio is at least 50% higher. In other cases, the increase in the RNA-SNP ratio is at least 100% higher. In still other cases, an RNA-SNP ratio that is at least 10% lower as compared to the average value in

the control sample, indicates an increased risk of having a fetus with a chromosomal disorder. In some other cases, the decrease is at least 50%, or at least 90%.

[0085] A RNA-SNP ratio that is at least one standard deviation higher or lower than the average value in the control sample indicates an increased risk of having a fetus with a chromosomal disorder. A RNA-SNP ratio that is at least two standard deviations higher or lower than the average value in the control sample indicates an increased risk of having a fetus with a chromosomal disorder. A RNA-SNP ratio that is at least three standard deviations higher or lower than the average value in the control sample indicates an increased risk of having a fetus with a chromosomal disorder. In some cases, a RNA-SNP ratio that is less than one standard deviation higher or lower than the average value in the control sample will also indicate an increased risk of having a fetus with a chromosomal disorder.

F. Kits for detecting a chromosomal disorder in a fetus of a pregnant woman

[0086] The present invention also provides kits for detecting the presence of a fetus having a chromosomal disorder. The kits of the present invention include primers for amplifying the region of interest. Primers useful in the kits of the present invention are described above for the discrimination of alleles. The primers can be specific for a marker or act in a non-specific fashion.

[0087] Another component of the kits of the present invention is a standard control representing the ratio of the RNA alleles in average pregnant women with a chromosomally normal fetus. The standard control is determined in the same manner as that described above for the methods of detecting the chromosomal disorder in the fetus of a pregnant woman.

[0088] The kits of the present invention can also comprise hybridization probes for discriminating the different alleles of each RNA species. The alleles of each RNA species can be single nucleotide polymorphisms, insertion/deletion polymorphisms or simple tandem repeat polymorphisms. Hybridization probes useful in the kits of the present invention are the same hybridization probes used for the methods of detecting the chromosomal disorder in the fetus of a pregnant woman. The hybridization probes can be radioactive, fluorescent, chemiluminescent or enzymatic. Oligonucleotides used as probes can be prepared according to known techniques (Beaucage and Caruthers, *Tetrahedron Letts.*, 22:1859-1862, 1981, using an automated synthesizer, as described in Needham-VanDevanter et al., *Nucleic Acids Res.* 12:6159-6168, 1984). The probes of the present invention can be specific or non-

specific. One of skill in the art will recognize that other methods of discriminating the alleles of each RNA species are useful in the kits of the present invention, such as those methods described above.

5 [0089] Kits of the instant invention may comprise additional elements that one of skill in the art will appreciate are useful in the kits of the present invention.

[0090] The following examples are provided by way of illustration only and not by way of limitation. Those of skill in the art will readily recognize a variety of non-critical parameters that can be changed or modified to yield essentially similar results.

III. Examples

10 [0091] The methods of the present invention are useful for detecting chromosomal disorders in individuals who are heterozygous for a particular genetic locus, to detect if the individuals have an abnormal number of chromosomes at that particular locus, e.g., an abnormal 3 instead of the normal 2. In the case of pregnancy, while the biological sample is taken from the pregnant woman directly, the individual being tested for the chromosomal
15 aneuploidy is the fetus that the pregnant woman is carrying. In one embodiment of the application of this approach for prenatal diagnosis, the extra copy of the gene is expressed along with the normal gene pair in placental tissues. The ratio of the RNA alleles in the placenta deviates from that of the normal placenta, as a result of the extra copy of the gene. The RNA transcripts are then released into the maternal blood, and their relative abundance is
20 reflective of the placental gene expression profile. Accordingly, the ratio of the RNA alleles in the blood or its fractions (e.g. plasma) of the pregnant woman carrying a fetus having a chromosomal disorder, deviates from that of a pregnant woman carrying a euploid fetus (Figure 1).

[0092] The detection of a chromosomal disorder using the RNA alleles requires the
25 identification of a marker that is specific for the fetus and is present at detectable expression levels in the mother's blood, identification of a transcribed region on that marker having high placental expression, the ability to detect the transcript in the mother's blood, determination that the transcripts are pregnancy-specific, and determination of the allelic-ratio of the transcripts in order to assess the presence or absence of the chromosomal disorder.

30 [0093] The examples below demonstrate specific examples of identifying useful markers and transcripts, methods for detecting the transcripts and ensuring that the transcripts are

pregnancy-specific, and methods for quantifying the transcripts and determining the RNA allele ratio to detect a chromosomal disorder. One of skill in the art will recognize that other methods and techniques are also useful in practicing the instant invention.

EXAMPLE 1: IDENTIFICATION OF SNPS USEFUL FOR DETECTING TRISOMY 21

5 IN FETUSES

[0094] The identification of SNPs useful for the detection of fetal trisomy 21 requires the identification of an RNA species that is expressed by fetal cells and is present in detectable concentrations in the analyzed biological samples.

Identification of fetal-specific transcripts with high placental expression levels

10 [0095] Gene expression profiles of five first-trimester chorionic villus sample (CVS) samples were obtained by microarray analysis of each individual tissue sample. In an effort to identify placenta-expressed transcripts amongst the circulating RNA molecules in maternal plasma, the gene expression profiles of maternal whole blood (specifically maternal hematopoietic cells) were obtained and compared with that of the corresponding placental
15 tissues. Placenta-expressed transcripts in early pregnancy were identified by selecting transcripts whose expression levels were increased in the CVS tissues when compared to the corresponding whole blood samples in all five comparisons.

[0096] *Sample processing and RNA extraction.* Five first-trimester placental tissue samples were obtained from pregnant women by CVS before therapeutic terminations. Fetal
20 karyotype in all cases were subsequently confirmed to be normal. The placental tissue samples were stored in RNeasy Lysis Buffer (Qiagen, Crawley, UK) immediately upon collection and kept at -80°C until RNA extraction. Six milliliters of maternal peripheral blood were collected concurrently at the time of tissue collection and stored in PAXgene™ Blood RNA Tubes (PreAnalytiX, Hombrechtikon, Switzerland). Total RNA from placental tissues were
25 extracted with the Trizol Reagent (Invitrogen, Carlsbad, CA) and purified with RNeasy mini-kit (Qiagen, Hilden, Germany) following manufacturer's protocols. Total RNA from peripheral blood was extracted by PAXgene™ Blood RNA Kit (PreAnalytiX, Hombrechtikon, Switzerland) according to manufacturer's instructions, with the inclusion of DNase treatment (RNase-Free DNase Set, Qiagen, Hilden, Germany).

30 [0097] *Gene expression analysis by high-density oligonucleotide arrays.* For each sample, ten micrograms of the extracted RNA were labeled and hybridized to the GeneChip® Human Genome U133A and U133B Arrays (Affymetrix, Santa Clara, CA) according to the

manufacturer's instructions. After hybridization, each array was washed and stained in a GeneChip® Fluidics Station 400 (Affymetrix). The chips were scanned with the GeneArray Scanner (Affymetrix) and analyzed using the GeneChip® Microarray Suite 5.0 (Affymetrix). Transcripts that were predominantly expressed in placental tissues instead of maternal peripheral blood and are derived from chromosome 21 were selected. Out of 7226 gene transcripts expressed in the CVS samples, 1245 transcripts were identified to have higher expression in the CVS than the maternal peripheral blood samples. Among this panel of transcripts, 13 placenta expressed genes located on chromosome 21 were identified (Table 1).

Table 1. Microarray detection of placenta-expressed genes located on chromosome 21.

Transcripts are sorted in descending order according to the median of the respective microarray signals.

Probe Set ID	Sequence Derived From	Transcripts	Symbol	Location	*Signal (median)
213428_s_at	AA292373	Collagen, type VI, alpha 1	COL6A1	21q22.3	8419.2
200642_at	NM_000454.1	superoxide dismutase 1, soluble (amyotrophic lateral sclerosis 1 (adult))	SOD1	21q22.11	7084.7
209156_s_at	AY029208.1	Collagen, type VI, alpha 2	COL6A2	21q22.3	7076.9
200818_at	NM_001697.1	ATP synthase, H ⁺ transporting, mitochondrial F1 complex, O subunit (oligomycin sensitivity conferring protein)	ATP5O	21q22.11	3247.8
213134_x_at	AI765445	BTG family, member 3	BTG3	21q21.1	2564.9
214953_s_at	X06989.1	amyloid beta (A4) precursor protein (protease nexin-II, Alzheimer disease)	APP	21q21.3	2376.1
202325_s_at	NM_001685.1	ATP synthase, H ⁺ transporting, mitochondrial F0 complex, subunit F6	ATP5J	21q21.1	2303.1
214750_at	L13197	placenta-specific 4	<i>PLAC4</i>	21q22.3	2209.9
222162_s_at	AK023795.1	a disintegrin-like and metalloprotease (repolysin type) with thrombospondin type 1 motif, 1	ADAMTS1	21q21.2	1780.8
217867_x_at	NM_012105.1	beta-site APP-cleaving enzyme 2	BACE2	21q22.3	1093.4
221689_s_at	AB035745.1	Down syndrome critical region gene 5	DSCR5	21q22.2	900.7
209298_s_at	AF114488.1	intersectin 1 (SH3 domain protein)	ITSN1	21q22.1- q22.2	199.9
#232191_at	BC005107.1	hypothetical protein BC005107	LOC90625	21q22.3	6910.2

* Medians of microarray signals from five first trimester placental tissues

Transcripts that were detected by Human Genome U133B Arrays (Affymetrix). Transcripts without specification were detected by Human Genome U133A Arrays (Affymetrix)

Identification of applicable SNPs and determination of allele frequencies

[0098] SNPs in the transcribed regions of the selected chromosome 21 genetic loci with predominant placental tissue expression were identified from a public database. The allele frequencies of each SNP were then determined in both Chinese and Caucasian populations. SNPs with high heterozygosity rates were targeted.

[0099] Among the 13 chromosome-21 genes with predominant placental expression (Table 1), four of the genes with the highest placental expression levels, namely *collagen VI alpha 1* (*COL6A1*), *superoxide dismutase 1* (*SOD1*), *collagen VI alpha 2* (*COL6A2*) and *mitochondrial ATP synthase O subunit* (*ATP5O*), were selected for further evaluation. SNPs that are located within exons of these genes were selected in order to enable the study of mRNA polymorphisms. The allele frequencies of each of these coding polymorphisms were determined in 10 Chinese and 10 Caucasian buffy coat DNA samples by direct sequencing with the use of PCR primers flanking the corresponding SNPs (Table 2). Sequencing was performed using BigDye Terminator Cycle Sequencing v1.1 (Applied Biosystems, Foster City, CA) and a Model 3100 DNA Analyzer (Applied Biosystems).

Table 2. PCR primers for the direct sequencing of the four chromosome 21 genes.

Transcripts	Sequences (5' to 3')	
	Forward primer	Reverse primer
<i>COL6A1</i>	GGCTGACATCACCATCCTG	AGAGCAGCAGCCTCTTCTTG
	TTGGAAAGCCAGGACACAAC	TGAGGATTGGTGGGAAAAC
<i>SOD1</i>	TTTTCCACTCCCAAGTCTGG	CGACAGAGCAAGACCCTTTC
	TTGCAACACCAAGAAAAGC	TCTGGCAAATACAGGTCATTG
<i>COL6A2</i>	TCATCAACGTGGTCAACAGG	TCACTCTCGTGCTTCTCGTG
	GTGGACATCGTCTTCCTGCT	GTGGATGGCAGTGAGGTTGT
	AACGACAGTCTGCACGAGTC	CAGGTAGGTCAGGAGCCTTG
<i>ATP5O</i>	GGCCTGAGATTCTTCACTGC	AAAATTAGCGGGACATGGTG

[0100] The allele frequencies of the transcribed SNPs are shown in Table 3. SNPs which were heterozygous in at least 30% of one of the two populations were considered to be informative and were selected as targets for further assay development.

Table 3. Genotyping of the coding SNPs in 10 Chinese and 10 Caucasian unrelated individuals for *COL6A1*, *SOD1*, *COL6A2* and *ATP5O*.

<i>COL6A1</i>	dbSNP	1053312	1053315	13879	1053320	1053331	9254
	Allele	A/G	A/G	T/C	T/C	A/G	A/G
<i>Chinese</i>	f (minor allele)	0.20	0.20	0	0.20	0	0.10
	Heterozygosity	0.32	0.32	0	0.320	0	0.18
<i>Caucasian</i>	f (minor allele)	0.40	0.40	0.05	0.40	0.15	0.15
	Heterozygosity	0.48	0.48	0.10	0.48	0.26	0.26
<i>SOD1</i>	dbSNP	1804450	1804449	4804447	15012	1804448	
	Allele	T/C	T/C	T/C	G/A	G/T	
<i>Chinese</i>	f (minor allele)	0	0	0	0.20	0	
	Heterozygosity	0	0	0	0.32	0	
<i>Caucasian</i>	f (minor allele)	0	0	0	0.25	0	
	Heterozygosity	0	0	0	0.38	0	
<i>COL6A2</i>	dbSNP	1042917	2839114	3182348	1042930	6652	1043801
	Allele	A/G	A/G	T/C	A/G	T/C	A/G
<i>Chinese</i>	f (minor allele)	0.50	0.50	0	0	0	0
	Heterozygosity	0.50	0.50	0	0	0	0
<i>Caucasian</i>	f (minor allele)	0.45	0.50	0	0	0.15	0.15
	Heterozygosity	0.50	0.50	0	0	0.26	0.26
	dbSNP	3087667	1043962	1043985			
	Allele	C/T	G/A	T/C			
<i>Chinese</i>	f (minor allele)	0	0	0			
	Heterozygosity	0	0	0			
<i>Caucasian</i>	f (minor allele)	0	0	0			
	Heterozygosity	0	0	0			
<i>ATP5O</i>	dbSNP	4842	4591				
	Allele	A/G	C/T				
<i>Chinese</i>	f (minor allele)	0.15	0				
	Heterozygosity	0.26	0				
<i>Caucasian</i>	f (minor allele)	0.40	0				
	Heterozygosity	0.48	0				

The dbSNP accession numbers for each SNP locus are listed. For each SNP, the minor allele is listed first. "f (minor allele)" denotes the frequency of the minor allele.

Determination of detectability and pregnancy-specificity of selected transcripts

[0101] The four chromosome 21 transcripts with placenta expression can be detected in maternal plasma using real-time QRT-PCR assays developed for amplifying the non-polymorphic regions of the four transcripts. These transcripts are present in the plasma of pregnant women at significantly higher concentrations as compared to non-pregnant women. In addition, the concentration of the transcripts in the maternal plasma drops precipitously

following birth of the child. Accordingly, the placenta is a predominant source of these mRNA transcripts in maternal plasma.

[0102] **Sample collection and processing.** Whole blood samples from six non-pregnant women and ten first-trimester pregnant women were collected. Peripheral blood samples
5 from five third-trimester pregnant women before and at 24 hours after delivery were also recruited. Twelve milliliters of the blood samples were collected in EDTA tubes and were centrifuged at 1600 x g for 10 min at 4° C. Plasma was then carefully transferred into plain polypropylene tubes. The plasma samples were re-centrifuged at 16000 x g for 10 min at 4°
10 C. Supernatants were collected into fresh polypropylene tubes. RNA extraction from maternal plasma was performed by mixing 3.2 ml of plasma with 4 ml of Trizol LS reagent (Invitrogen, Carlsbad, CA) and 0.8 ml of chloroform (Ng, E. K. O. et al., *Clin Chem* 48:1212-1217 (2002)). The mixture was centrifuged at 12000 x g for 15 min at 4°C and the aqueous
15 layer was transferred into new tubes. One volume of 70% ethanol was added to one volume of the aqueous layer. The mixture was then applied to an RNeasy mini column (Qiagen, Hilden, Germany) and was processed according to manufacturer's recommendations. Total RNA was eluted with 60 µl of RNase-free water and stored at -80°C. DNase treatment (RNase-Free DNase Set, Qiagen, Hilden, Germany) was carried out to remove any
contaminating DNA.

[0103] **Development of real-time QRT-PCR assays.** QRT-PCR assays were developed for
20 the detection of *collagen VI alpha 1 (COL6A1)*, *superoxide dismutase 1 (SOD1)*, *collagen VI alpha 2 (COL6A2)* and *mitochondrial ATP synthase O subunit (ATP5O)* mRNA. The sequences of the primers and the TaqMan minor-groove-binding (MGB) fluorescent probes (Applied Biosystems, Foster City, CA, USA) are shown in Table 4. Calibration curves were prepared by serial dilutions of high performance liquid chromatography-purified single
25 stranded synthetic DNA oligonucleotides (Bustin, 2000) (Proligo, Singapore) specific for the respective amplicons, with concentrations ranging from 1 X 10⁶ copies to 10 copies. The sequences of the synthetic DNA oligonucleotide for *COL6A1*, *SOD1*, *COL6A2* and *ATP5O* are described in Table 4. Absolute concentrations of all transcripts were expressed as copies/ml of plasma.

Table 4. Sequences of primers and probes for real-time QRT-PCR detection of the placenta-expressed transcripts encoded on chromosome 21.

Transcripts		Sequences (5' to 3') ¹
COL6A1	F primer	GACAAAGTCAAGTCCTTCACCAA
	R primer	GCGTTCCACACCAGGTTT
	Probe	(FAM) CGCTTCATCGACAACC (MGBNFQ)
	standard curve	TGGACAAAGTCAAGTCCTTCACCAAGCGCTTCATCGACAACCTGAGGGACAGGTACTACCG CTGTGACCGAAACCTGGTGTGGAACGCAG
SOD1	F primer	CAGGGCATCATCAATTTTCG
	R primer	TGCTTCCCCACACCTTCA
	Probe	(FAM) CAGAAGGAAAGTAATGGACCA (MGBNFQ)
	standard curve	TGCAGGGCATCATCAATTTTCGAGCAGAAGGAAAGTAATGGACCAGTGAAGGTGTGGGGAAG CATT
COL6A2	F primer	GATCAACCAGGACACCATCAA
	R primer	CCGTAGGCTTCGTGTTTCA
	Probe	(FAM) CGCATCATCAAGGTC (MGBNFQ)
	standard curve	GAGATCAACCAGGACACCATCAACCGCATCATCAAGGTCATGAAACACGAAGCCTACGGAG
ATP5O	F primer	CCCTCACTACCAACCTGATCA
	R primer	CCTTGGGTATTGCTTAATCGA
	Probe	(FAM) TGCTTGCTGAAAATG (MGBNFQ)
	standard curve	TCCCCTCACTACCAACCTGATCAATTTGCTTGCTGAAAATGGTCGATTAAGCAATACC CAAGGAG

¹ FAM: fluorescent label; MGBNFQ: minor groove binding non-fluorescent quencher.

5 [0104] The QRT-PCR reactions were set up according to the manufacturer's instructions (EZ *rTth* RNA PCR reagent set, Applied Biosystems) in a reaction volume of 25 µl. The QRT-PCR assays were carried out in a combined thermal cycler and fluorescent detector (ABI Prism 7700, Applied Biosystems). For all of the four transcripts, the PCR primers (Proligo) and the fluorescent probes (Applied Biosystems) were used at concentrations of 300
10 nM and 100 nM, respectively. 5 µl of extracted plasma RNA were used for amplifications. Multiple negative water blanks were included in every analysis.

[0105] The thermal profiles used for *COL6A1*, *SOD1*, *COL6A2* and *ATP5O* analysis were as follows: the reaction was initiated at 50°C for 2 min for the included uracil N-glycosylase to act, followed by reverse transcription at 60°C for 30 min. After a 5-min denaturation at 95
15 °C, 40 cycles of PCR were carried out using denaturation at 92°C for 15 s and 1 min annealing/extension at 57°C for *COL6A1* and *ATP5O*, 56°C for *COL6A2* and 59°C for *SOD1*.

[0106] *Placenta-expressed chromosome 21 transcripts can be detected in maternal plasma and are pregnancy-specific.* The median plasma mRNA concentrations in non-

pregnant women, first trimester and third trimester pregnancies were 0 copies/ml, 0 copies/ml and 72.6 copies/ml, respectively, for *COL6A1* (Figure 2A); 25.3 copies/ml, 53.0 copies/ml and 155.6 copies/ml for *SOD1* (Figure 2B); 0.8 copies/ml, 2.1 copies/ml and 8.8 copies/ml for *COL6A2*, (Figure 2C); and 6.2 copies/ml, 88.2 copies/ml and 126.4 copies/ml for *ATP5O* (Figure 2D). For all of the four transcripts, their plasma concentrations in the third trimester of pregnancy were significantly higher than that in the non-pregnant group (Mann-Whitney Rank Sum test, $P < 0.05$ for *COL6A1*, *SOD1*, *COL6A2* and *ATP5O* mRNA). Furthermore, in the pre-delivery plasma samples, the median *COL6A1*, *SOD1*, *COL6A2* and *ATP5O* mRNA concentrations were 72.6 copies/ml, 155.6 copies/ml, 8.8 copies/ml and 126.4 copies/ml, respectively (Figure 3A for *COL6A1* mRNA, Figure 3B for *SOD1* mRNA, Figure 3C for *COL6A2* mRNA and Figure 3D for *ATP5O* mRNA). While in the postpartum plasma samples, the median mRNA concentrations of *COL6A1*, *SOD1*, *COL6A2* and *ATP5O* were 0 copies/ml, 56.2 copies/ml, 0.8 copies/ml and 56.2 copies/ml, respectively.

EXAMPLE 2: DETECTION OF TRISOMY 21 IN THE FETUS OF A PREGNANT WOMAN BY REAL-TIME QUANTITATIVE RT-PCR

[0107] The two SNPs with the highest polymorphic rates were selected as targets for allele-specific QRT-PCR development. Discriminative hybridization probes were designed to allow discrimination of the different alleles of each SNP. Identical genotyping results were obtained by both the real-time PCR and direct sequencing which confirmed the allele-specificity of the probes. The probes were then incorporated into QRT-PCR assays where the relative expression levels of the different alleles of each SNP in placental tissues were first measured. RNA extracted from normal CVS and normal term placentas, as well as from placentas of trisomy 21 pregnancies were assayed by the allele-specific QRT-PCR. The ratios of the alleles of each SNP in the pregnancies with and without trisomy 21 are substantially different, enabling the detection of trisomy 21 in the fetus using the mother's plasma.

[0108] **Sample collection and processing.** Placental tissue samples from 13 first trimester and 20 third trimester pregnancies were obtained from pregnant women by chorionic villus sample (CVS) before therapeutic terminations or immediately after elective cesarean delivery, respectively. Placental tissues from seven trisomy 21 pregnancies were also recruited. All tissues were processed as described above.

[0109] **Development of allele-specific real time QRT-PCR.** The two informative coding SNPs with the highest polymorphic rates, rs1053312 (dbSNP Accession numbers) and rs2839114 of the COL6A1 and COL6A2 genes, respectively, were selected. Allele-specific real-time quantitative RT-PCR assays were established for measuring the relative concentrations of the two alleles of each SNP. To allow allelic discrimination, two fluorogenic MGB probes, each specific for one allele, are included in each QRT-PCR assay. The two probes are labeled with either FAM (6-carboxyfluorescein) and VIC fluorescent reporter dyes. The sequences of the primers and probes of the allele-specific QRT-PCR systems are shown in Table 5. Identical genotyping results were obtained by both the real-time PCR and direct sequencing which confirmed the allele-specificity of the probes.

Table 5. Sequences of primers and probes for allele-specific real-time QRT-PCR detection of the targeted RNA-SNPs.

Transcripts	dbSNP		Sequences (5' to 3') ¹
COL6A1	1053312	F primer	GGCAGCCACAACCTTTGACAC
		R primer	CTCGGCCAGGCGCTT
		Probe (allele G)	(VIC) ACCAAGCGCTTCGC (MGBNFQ)
		Probe (allele A)	(FAM) ACCAAGCACTTCGC (MGBNFQ)
COL6A2	2839114	F primer	GGCGCCAGAAGACACGT
		R primer	GTCGTGGCGCCCGT
		Probe (allele G)	(VIC) TGATGACCACCGCAAA (MGBNFQ)
		Probe (allele A)	(FAM) TGATGACTACCGCAAA (MGBNFQ)

¹ VIC and FAM: fluorescent labels; MGBNFQ: minor groove binding non-fluorescent quencher.

15

[0110] The QRT-PCR reactions for allele discrimination and relative quantification were set up according to the procedure set forth above using the PCR primers (Proligo) and the MGB probes (Applied Biosystems) at concentrations of 450 nM and 100 nM, respectively, and 17 ng of placental RNA samples for amplifications. Each QRT-PCR was performed in duplicate in an Applied Biosystems 7700 Sequence Detector. The thermal profile used was that described above using a temperature of 59° C for the annealing/extension step. Only heterozygous samples were included in the analysis.

20

[0111] **Statistical Analysis.** Statistical analysis was performed using the Sigma Stat 2.03 software (SPSS).

[0112] *Relative quantification of chromosome 21 encoded RNA-SNPs.* The relative amounts of the two alleles are determined either by the difference in their threshold cycle values (ΔC_t) or by the difference in their accumulated fluorescent intensities ($\Delta \Delta R_n$), as calculated by the following equations:

$$\Delta C_t = C_{t_{FAM}} - C_{t_{VIC}}$$

$$\Delta \Delta R_n = \Delta R_{n_{FAM}} - \Delta R_{n_{VIC}}$$

where $C_{t_{FAM}}$ and $C_{t_{VIC}}$ are the threshold cycle values of allele A (as detected by a FAM-labeled probe) and allele B (as detected by a VIC-labeled probe). $\Delta R_{n_{FAM}}$ and $\Delta R_{n_{VIC}}$ are the accumulated fluorescent intensities of allele A and allele B, as calculated by the SDS v1.9 software (Applied Biosystems). The C_t and ΔR_n values are logarithmically related to the abundance of PCR products, thus the difference in the C_t and ΔR_n values for each RNA-SNP (the ΔC_t and $\Delta \Delta R_n$ values) is reflective of the RNA-SNP ratio between the two RNA alleles.

[0113] For the SNP rs1053312 (*COL6A1*), one CVS, six term placentas from normal pregnancies and three trisomy 21 placenta samples were heterozygous for the polymorphic site, as determined by the allele-specific QRT-PCR. In Figure 4, the ΔC_t values for the three trisomy 21 placentas deviated from the ΔC_t values of the CVS and term placental tissues obtained from normal pregnancies.

[0114] For the SNP rs2839114 (*COL6A2*), eight CVS and thirteen term placentas from normal pregnancies, as well as three trisomy 21 placentas were heterozygous for the SNP locus as genotyped by the allele-specific QRT-PCR assay. As shown in Figure 5A, the ΔC_t values of the three trisomy 21 placentas deviated from those of the normal pregnancies, with two trisomy 21 cases exhibiting greater ΔC_t values and the remaining case with a reduced value. The deviations of the ΔC_t values of trisomy 21 placentas may fall either above or below the normal pregnancy range, dependent on which allele is overrepresented. Similar results were obtained by using the $\Delta \Delta R_n$ values (Figure 5B).

EXAMPLE 3: DETECTION OF TRISOMY 21 IN THE FETUS OF A PREGNANT WOMAN BY PRIMER EXTENSION AND MASS SPECTROMETRY DETECTION

[0115] The two SNPs with the highest polymorphic rates (rs1053320 on *COL6A1* and rs2839114 on *COL6A2*) were targeted for further assay development. Primer extension reaction assays enabled determination of the SNP genotype of the placental tissue samples. The samples were processed using mass spectrometry to differentiate the different RNA-SNP

alleles, and determine the relative expression levels of the RNA-SNP alleles in order to calculate the ratio of alleles. The difference in the ratios of the alleles of each SNP for pregnancies with and without trisomy 21 is sufficiently large that detection of trisomy 21 in the fetus is possible using the mother's plasma.

5 [0116] **Placental tissue collection and processing.** Second-trimester placental tissue samples were obtained from pregnant women carrying trisomy 21 fetuses before therapeutic terminations. Third-trimester placental tissue samples were collected from karyotypically normal fetuses immediately after delivery. The placental tissue samples were divided into two portions, one of which was stored in RNAlater™ (Ambion®, Austin, TX) immediately
10 upon collection and kept at -80° C until RNA extraction, while the other portion was immediately kept at -80° C until DNA extraction. DNA was extracted from the placental tissues using the QIAamp mini kit using the tissue protocol according to the manufacturer's instructions (Qiagen, Hilden, Germany). Total RNA from placental tissues were extracted with the Trizol Reagent (Invitrogen, Carlsbad, CA) and purified with RNeasy mini-kit
15 (Qiagen, Hilden, Germany) following manufacturer's protocols.

[0117] **Placental DNA and RNA amplification.** For RNA amplification, a total of 450 ng of placental RNA was reverse transcribed with random hexamers (TheromScript, Invitrogen) according to manufacturer's instruction. 25 ng of DNA or cDNA corresponding to 50 ng of total RNA was used for each PCR reaction (AmpliTaq Gold, Applied Biosystems). The
20 primer sequences for the amplification of each of the SNP locus on *COL6A1* and *COL6A2* are shown in Table 6. PCR primers were used at 200 nM final concentrations for a PCR volume of 25 µl. The PCR condition was: 95° C for 10 min, followed by denaturing at 94° C for 20 sec, annealing at 56° C for 30 sec, and extension at 72° C for 1 min for 45 cycles, and finally incubation at 72° C for 3 min.

25 **Table 6.** Primer sequences for the amplification of the SNP loci on *COL6A1* and *COL6A2*.

Transcripts	SNP		Sequences (5' to 3')
<i>COL6A1</i>	rs1053320	F primer	ACGTTGGATGCTATGTGACCCGCTTCTACC
		R primer	ACGTTGGATGGAGTTGCCATCTGAGAAGAG
<i>COL6A2</i>	rs2839114	F primer	ACGTTGGATGACCGCCTCATCAAGGAGAGC
		R primer	ACGTTGGATGAAGTTGAGGTCATCGTCCCG

[0118] **SNP detection by primer extension reaction.** Primer extension reaction assays were designed to determine the SNP genotype of the placental tissue samples. The placental RNA-SNP genotypes were compared with that obtained for placental DNA. The placental DNA

and RNA PCR products were treated with shrimp alkaline phosphatase (Sequenom, San Diego, USA) for 40 min at 37° C followed by 5 min at 85° C to remove excess dNTPs. A primer extension primer and a mixture of 2',3'-dideoxynucleoside triphosphates (ddNTPs) and dNTPs were added to the treated PCR products. Sequences for the primer extension primer designed to interrogate the chosen SNP on *COL6A1* and *COL6A2* are shown in Table 7. A thermoSequenase (Sequenom) was used for the base extension reactions using standard MassARRAY™ Homogeneous MassEXTEND™ (hME) assay protocol from Sequenom. The base extension condition was: 94° C for 2 min, followed by 94° C for 5 sec, 52° C for 5 sec, and 72° C for 5 sec for 75 cycles. Sequences of the primer extended products for each SNP allele are shown in Table 7. The final extension products were treated with SpectroCLEAN (Sequenom) resin to remove salts in the reaction buffer. Approximately 10 nL of reaction solution was dispensed onto a 384-format SpectroCHIP (Sequenom) using a SpectroPoint (Sequenom) nanodispenser. A compact MALDI-TOF mass spectrometer (Bruker) was used for data acquisitions. The expected molecular weights of all relevant peaks are calculated before the analysis (Table 7) and identified from the mass spectrum. The SNP genotype is determined by scoring the presence or absence of the mass signals corresponding to a particular SNP allele.

Table 7. Sequences of the primer extension primers and expected sequences and molecular masses of the extended products for the respective SNP alleles for rs1053320 on *COL6A1* and rs2839114 on *COL6A2*.

Transcripts	SNP		Extended Sequences ¹	Mass (Da)
<i>COL6A1</i>	rs1053320	un-extended	CTCTTCTTGGCAGCGCC	5113.3
		allele T	CTCTTCTTGGCAGCGCCA	5410.5
		allele C	CTCTTCTTGGCAGCGCC GGA	6068.9
<i>COL6A2</i>	rs2839114	un-extended	AAGACACGTGTGTTTGCGGT	6188.0
		allele A	AAGACACGTGTGTTTGCGG TA	6485.2
		allele G	AAGACACGTGTGTTTGCGGT GGT	7134.6

¹ Bold fonts indicate the extended dNTPs and ddNTPs.

[0119] **Determination of SNP ratio.** Mass spectrometric data were automatically imported into the SpectroTYPER (Sequenom) database for automatic analysis. The ratio of the peak frequency of the mass signals for the two SNP alleles in fetuses heterozygous for the interrogated SNP was determined. The SNP ratio obtained for karyotypically normal and trisomy 21 fetuses were compared. The placental DNA SNP ratio of the trisomy 21 fetuses deviated from that obtained for the normal fetuses for the SNPs on both *COL6A1* (Figure 6A) and *COL6A2* (Figure 7A). The SNP-ratio for the trisomy 21 fetuses would either be

decreased or increased compared with that of the normal fetuses depending on the SNP genotype of the trisomic chromosome 21. The placental tissue SNP ratio of the interrogated SNPs on *COL6A1* (Figure 6B) and *COL6A2* (Figure 7B) RNA transcripts for the trisomy 21 fetuses was also shown to deviate from that obtained for normal fetuses.

5

EXAMPLE 4: NONINVASIVE PRENATAL DETECTION OF TRISOMY 21 FETUS USING CIRCULATING FETAL RNA IN MATERNAL PLASMA BY PRIMER EXTENSION AND MASS SPECTROMETRY

[0120] In order to demonstrate that additional SNPs are also useful for the detection of chromosomal disorders in the fetus of a pregnant woman, *placenta-specific 4 (PLAC4)* (Table 19) was investigated. Primer extension reaction assays enabled determination of the RNA-SNP genotype of the placental tissue and maternal plasma samples. The primer extension products were analyzed using mass spectrometry to quantify the RNA-SNP allele ratios. The difference in the ratios of the alleles of a *PLAC4* SNP for pregnancies with and without trisomy 21 is sufficiently great so that detection of trisomy 21 of the fetus is possible using the mother's plasma.

Identification of applicable coding SNPs in *placenta-specific 4 (PLAC4)* gene and determination of allele frequencies

[0121] **SNP identification.** The *placenta-specific 4 (PLAC4)* gene is highly expressed in the placenta, but expressed at low levels in the buffy coat cells. The *PLAC4* gene is located in the Down syndrome critical region of chromosome 21. The *PLAC4* gene sequence is listed in Table 19 and spans the nucleotide coordinates 41469028-41480585 on chromosome 21 based on the Human May 2004 (hg17) assembly at the UCSC Genome Browser (a website having a database of these gene sequences and nucleic acid sequences for genomes

offered at the time of preparation of this application based in the U.S.). The *PLAC4* gene sequence as listed in Table 19 consists of all known and predicted *PLAC4* RNA splicing variants identified by the GenBank accession numbers: AF269287, AK027868, AK092431, BC093685, BC101615, BC101617, L13197, NM 182832 and LOC191585. Polymorphic SNPs were identified by sequencing the exonic/transcribed regions of the *PLAC4* gene. Direct sequencing was performed on placental DNA samples from 10 unrelated Chinese pregnant women. Twenty nanograms of genomic DNA was first amplified by PCR. Sequencing was performed using BigDye Terminator Cycle Sequencing v1.1 (Applied Biosystems, Foster City, CA) and a Model 3100 DNA Analyzer (Applied Biosystems).

[0122] **Allele frequency determination.** Four coding SNPs in the transcribed region of *PLAC4* were found to be polymorphic in the Chinese population (Table 8). These four SNPs are located at the chromosome 21 nucleotide positions 41470591, 41471145, 41476236 and 41478755, respectively, of the chromosome 21 sequence of the Human May 2004 (hg17) assembly at the UCSC Genome Browser. The dbSNP accession numbers for the SNPs found at the chromosome 21 nucleotide positions 41470591 and 41478755 are rs9977003 and rs8130833, respectively. The remaining two SNPs were novel ones which are hereby named as *PLAC4*-41471145 and *PLAC4*-41476236, according to their nucleotide coordinates on chromosome 21 on the Human May 2004 (hg17) assembly at the UCSC Genome Browser. Their allele frequencies are shown in Table 9. rs8130833 was the most polymorphic SNP among the four SNPs and was selected for further assay development.

Table 8. Four polymorphic SNPs located in the coding region of *PLAC4*.

SNP	Sequence
<i>PLAC4</i> - 41478755 ¹ (rs8130833) ²	5' - TTTTACAAAATAAGCCTAATCGTAAATATCACTATAGTATATAGAACCATGTTTAGGCC AGATATATTCGTC [A/G] TCTAACTTGTATTTAACCCAAATGGTGTGCAATACAAAATGAGTT TCTTTTCTTTAAGCCAAATTTGAATTTGCTCCAATAGCTTAAAAGACACCCTAGCGGCG -3'
<i>PLAC4</i> - 41471145 ¹	5' - CTCACATCTCTAAAGGCCACTCAGGTGGGACACCATCAAGACATTGAAAATCGACAGAAG GAAGGCAGGAAGGGGAGAGGATC [A/G] AACCTGTCAAAATAGATATTCAGAAAATCTGTGCTC TAAAATAAGGCAGCCCTTCCCTCACAGCACACTTATTCCTAATTTCAACAGGACTCCTAG -3'
<i>PLAC4</i> - 41470591 ¹ (rs9977003) ²	5' - TCATTCTGAGGCGGTGCTGCTGAAAATCTTGGTGCTGAACGTGTGTTTTTGAGATTTCCA GTCTATCAC [A/G] GGGCCACAAGGTGTAAATATCAAGAAAAATGAATTACTAGAAAGGCAAAG TGAAAAAGACATACAAAATACAAGTGTCAATCTTTTTTATTCTTAGTTTGGACAGATAGTC -3'
<i>PLAC4</i> - 41476236 ¹	5' - TAATTGATTCTTGGGGTATCCCTGTCTTTACCCTGTCTTTAAGGTTTTAGCAAGACTAAG TCTCCTGGTTGAAC [C/T] GGGGAGCTATTTTTCCTTTGTGGGGAAGGACAGTATTTTATTTT TATATTGGAGGGCCTTTTGAACCTGTCCTAAATCTAAAGGGGAGGGGGGAAGGTTTCATA -3'

The *PLAC4* mRNA was transcribed in the reverse direction of the sequences showed in the table.

¹ The number marks the location of the SNP based on the Human May 2004 (hg17) assembly at the UCSC Genome Browser.

² dbSNP accession number

Table 9. Allele frequencies of four *PLAC4* coding SNPs in placental DNA samples of 10 unrelated Chinese pregnant women.

SNP	rs8130833	<i>PLAC4</i> - 41471145	rs9977003	<i>PLAC4</i> - 41476236
Allele	G/A	A/G	A/G	T/C
f (minor allele)	0.25	0.10	0.10	0.10
Heterozygosity	0.38	0.18	0.18	0.18

For each SNP, the minor allele is listed first. "f (minor allele)" denotes the frequency of the minor allele.

5

Determination of allelic ratios of *PLAC4* RNA in maternal plasma using a SNP marker

[0123] **Sample collection and processing.** First- and second-trimester placental tissue samples were obtained from seven pregnant women carrying trisomy 21 fetuses. Placental tissues from 26 pregnant women carrying karyotypically normal fetuses were also collected by chorionic villus sampling (CVS). The placental samples were stored in RNAlaterTM (Ambion[®], Austin, TX) immediately upon collection and kept at -80°C until RNA extraction. Peripheral blood samples were collected from 43 pregnant women carrying a single euploid fetus and five pregnant women carrying a single trisomy 21 fetus during their first-trimester of pregnancy. Plasma samples were harvested as described in Example 1.

[0124] Total RNA from the placental tissues was extracted with Trizol (Qiagen, Hilden, Germany) following the manufacturer's protocol. RNA was extracted from 1.6 to 3.2 ml of the maternal plasma samples. For every milliliter of plasma, 3 ml of Trizol LS reagent and 0.8 ml of chloroform were added. The mixture was centrifuged at 12,000 x *g* for 15 min at 4°C. After centrifugation, the aqueous layer was collected. Five hundred and thirty-eight microliters of absolute ethanol was added per 1 ml of aqueous layer. The mixture was applied to the RNeasy mini columns (Qiagen, Hilden, Germany) and were processed according to manufacturer's recommendations. Total RNA was eluted with 48 µl of RNase-free water for each column. The final eluted RNA from the two columns was pooled together. DNase treatment was then carried out to remove any contaminating DNA (Invitrogen, Carlsbad, CA, USA).

[0125] **Reverse transcription and PCR amplification.** 1.25 micrograms of placental RNA or 48 µL of plasma RNA was reverse transcribed in a reaction volume of 40 µl or 100 µl, respectively, according to manufacturer's instructions (ThermoScript, Invitrogen, Carlsbad, CA, USA), using gene-specific primers (sequences are shown in Table 10).

[0126] For each PCR amplification reaction, 40 μ l of the placental cDNA or 100 μ l of the maternal plasma cDNA was used in a total volume of 80 μ l or 200 μ l, respectively. Each reaction contained 0.6X HotStar *Taq* PCR buffer with 0.9 mM $MgCl_2$ (Qiagen), 25 μ M each of dATP, dGTP and dCTP, 50 μ M of dUTP (Applied Biosystems), 200 nM each of forward and reverse primers (Integrated DNA Technologies) and 0.02 U/ μ l of HotStar *Taq* Polymerase (Qiagen). The PCR primer sequences are shown in Table 10. The PCR reaction was initiated at 95°C for 7 min, followed by denaturation at 95°C for 40 sec, annealing at 56°C for 1 min, extension at 72°C for 1 min for 55 cycles, and a final incubation at 72°C for 3 min.

10 **Table 10.** Primer sequences for reverse transcription and PCR amplification of the sequence containing SNP rs8130833.

Primer	Sequence (5' to 3')
Gene-specific primer for reverse transcription	GTATATAGAACCATGTTTAGGCCAG
Forward PCR primer	ACGTTGGATGGTATTGCAACACCATTTGGG
Reverse PCR primer	ACGTTGGATGTAGAACCATGTTTAGGCCAG

[0127] *SNP detection and allelic ratio quantification by primer extension reaction.* The primer extension reaction was performed as was described in Example 3. The PCR product was first treated with shrimp alkaline phosphatase (Sequenom, San Diego, USA). Four microliters of base extension cocktail containing 771 nM of extension primer (Integrated DNA Technologies), 1.15U of Thermosequenase (Sequenom) and 64 μ M each of ddATP, ddCTP, ddTTP and dGTP (Sequenom, San Diego, USA) were mixed with 5 μ l of water and 5 μ l of the PCR product. The thermal profile was 94°C for 2 min, followed by 94°C for 5 sec, 52°C for 5 sec, and 72 °C for 5 sec for 100 cycles. Sequences and molecular weights for the extension primer and the extension products for each SNP allele are shown in Table 11. The molecular weights of the final extension products were determined by MALDI-TOF mass spectrometer as was described in Example 3. The ratios of the peak areas for the primer extension products representing the two SNP alleles in fetuses heterozygous for the SNP were determined.

Table 11. Sequences and molecular weights of the extension primer and the expected extension products for SNP rs8130833.

	Sequence	Molecular Weight (Da)
Unextended primer	AGGCCAGATATATTCGTC	5498.6
Extension product for allele A	AGGCCAGATATATTCG TCA	5795.8
Extension product for allele G	AGGCCAGATATATTCG TCGT	6116.0

Letters in bold font indicate the dNTPs and ddNTPs residues added to the extension primer.

5 Development of real-time QRT-PCR assay

10 [0128] A QRT-PCR assay for *PLAC4* mRNA was developed to assess if there are quantitative differences in maternal plasma *PLAC4* mRNA concentrations between trisomy 21 and normal pregnancies. The sequences of the primers (Integrated DNA Technologies, Coralville, IA), TaqMan minor groove binding (MGB) fluorescent probes (Applied Biosystems, Foster City, CA, USA) and the calibrator (Proligo, Singapore) are shown in Table 12.

Table 12. Sequences of the PCR primers, the probe and the calibrator for real-time QRT-PCR detection of *PLAC4* mRNA.

Primer	Sequence (5' to 3')
F primer	CCTTTCCCCCTTATCCA ACT
R primer	G T ACTGGTTGGGCTCATT TT CT
Probe	(FAM) CCCTAGCCTATACCC (MGBNFQ)
Calibrator	CACCTTTCCCCCTTATCCA ACT AGCCCTAGCCTATAC CT CTGCTGCCCA AGAAAATGAGCCCAACCAGTACAC

MGBNFQ: minor groove binding non-fluorescent quencher

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[0129] The QRT-PCR reactions were set up according to the manufacturer's instructions (EZ *rTth* RNA PCR reagent set, Applied Biosystems) in a reaction volume of 25 μ l. The QRT-PCR assays were carried out in an ABI PRISM[®] 7900HT (Applied Biosystems, Foster City, CA, USA). The PCR primers and the fluorescent probe were used at concentrations of 20 400 nM and 100 nM, respectively. 5 μ l of extracted RNA were used for amplification. The thermal cycling profile was: the reaction was initiated at 50°C for 2 min, followed by reverse

transcription at 60°C for 30 min. After a 5-min denaturation at 95°C, 45 cycles of PCR were carried out using denaturation at 95°C for 15s and 1 min at 60°C.

Detection of difference in *PLAC4* RNA allelic ratio in placentas and maternal plasma samples from pregnant women with trisomy 21 and normal fetuses

5 [0130] The RNA transcript allelic ratios in the placentas of karyotypically normal and trisomy 21 pregnancies were compared using the SNP rs8130833. The ratios were calculated by dividing the relative amount of allele G (higher-mass allele, i.e., the allele whereby the extension product demonstrates a higher mass in the mass spectra) to the relative amount of
10 allele A (lower-mass allele, i.e., the allele whereby the extension product demonstrates a lower mass in the mass spectra). As shown in Figure 8, all trisomy 21 samples showed allelic ratios well distinguishable from the normal samples. The allelic ratios of the trisomy 21 samples segregated into two groups. Trisomy samples with an extra G allele showed an allelic ratio higher than the normal range, while samples with an extra A allele showed an allelic ratio lower than the normal range.

15 [0131] The *PLAC4* mRNA allelic ratios were also compared in maternal plasma from women carrying karyotypically normal and trisomy 21 fetuses using the SNP rs8130833. All but one trisomy sample demonstrated allelic ratios that deviated from the normal samples (Figure 9). The data show that fetal aneuploidy can be detected non-invasively by analyzing the allelic ratios of circulating fetal-specific transcripts in maternal plasma by using
20 polymorphic markers such as SNP.

[0132] A positive correlation was found for the SNP rs8130833 allelic ratio between the placental *PLAC4* RNA and the circulating *PLAC4* RNA in the maternal plasma (Figure 10) (Pearson correlation, $P < 0.05$). This finding provides additional evidence that the placenta is a major source for releasing *PLAC4* mRNA into maternal plasma.

25 Comparison of Circulating *PLAC4* mRNA in Euploid and Trisomy 21 Pregnancies

[0133] Circulating *PLAC4* mRNA concentrations were compared between karyotypically normal and trisomy 21 pregnancies. Plasma samples were collected from 29 pregnant women carrying euploid fetuses and five pregnant women carrying trisomy 21 fetuses during
the first- and second- trimester of pregnancy. The plasma samples were measured for *PLAC4*
30 mRNA concentrations by real-time one-step RT-PCR as described. As shown in Figure 11, *PLAC4* mRNA was detected in all of the trisomic plasma samples. The medians for the trisomy 21 and normal pregnancies are 5581 copies/ml and 4836 copies/ml, respectively. No

statistically significant difference was established for the plasma *PLAC4* mRNA concentrations between the normal and the trisomy 21 pregnancies. This demonstrates that the mere quantification of *PLAC4* mRNA in maternal plasma does not provide a reliable assessment of the presence of fetal trisomy 21.

5

EXAMPLE 5: DETECTION OF TRISOMY 18 FETUS USING FETAL-EXPRESSED RNA IN PLACENTAS BY PRIMER EXTENSION AND MASS SPECTROMETRY

[0134] In order to demonstrate that additional chromosomal disorders can be detected using other genes, *serpin peptidase inhibitor clade B (ovalbumin) member 2 (SERPINB2)* (GenBank Accession number: NM_002575) was investigated for the ability to detect trisomy 18. Primer extension reaction assays enabled determination of the SNP genotype of the placental tissue samples. The samples were processed using mass spectrometry to differentiate the different RNA-SNP alleles, and determine the relative expression levels of the RNA-SNP alleles in order to calculate the ratio of alleles. The difference in the ratios of the alleles of a *SERPINB2* SNP for pregnancies with and without trisomy 18 is sufficiently great that detection of trisomy 18 in the fetus is possible using placental RNA samples.

Determination of allelic ratio of serpin peptidase inhibitor clade B (ovalbumin) member 2 mRNA in placentas

[0135] The placental-expressed *serpin peptidase inhibitor clade B (ovalbumin) member 2 (SERPINB2)* was chosen. The *SERPINB2* gene is located on chromosome 18. A polymorphic SNP (Table 13) located within the coding region of *SERPINB2* gene was identified from a public database and was chosen for assay development.

Table 13. A polymorphic SNP within the coding region of *SERPINB2* gene

SNP	Sequence
SERPINB2 (rs6098) ¹	5' - GTTCTGTGTTATATATAAAGAATTCCTTCTTTCTTTTCAAGGCACAAGCTGCAGATAAAAT CCATTCATCCTTCCGCTCTCTCAGCTCTGCAATCAATGCATCCACAGGG [A/G] ATTATTTACTG GAAAGTGTCAATAAGCTGTTTGGTGAGAAGTCTGCGAGCTTCCGGGAAGTAAGTGAAACCTG - 3'

¹ dbSNP accession number

25

[0136] **Sample collection and processing.** First- and second-trimester placental tissue samples were obtained from four pregnant women carrying trisomy 18 fetuses. Placental samples from eight first-trimester pregnant women carrying karyotypically normal fetuses were also collected by chorionic villus sampling (CVS). The samples were stored in RNAlaterTM (Ambion®, Austin, TX) immediately upon collection and kept at -80°C until

30

RNA extraction. Total RNA was extracted using Trizol (Qiagen, Hilden, Germany) following the manufacturer's protocol. The extracted RNA sample was treated with DNase (Invitrogen, Carlsbad, CA, USA) to remove any contaminating DNA.

[0137] **Reverse transcription and PCR amplification.** 0.625 micrograms of placental RNA was reverse transcribed in a reaction volume of 20 μ l according to manufacturer's instructions (ThermoScript, Invitrogen, Carlsbad, CA, USA). The reverse transcription was performed using the reverse PCR primer with sequence shown in Table 14.

[0138] For PCR amplification, 20 μ l of the placental cDNA was used in a total reaction volume of 40 μ l. Each reaction contained 0.6X HotStar *Taq* PCR buffer with 0.9 mM $MgCl_2$ (Qiagen), 25 μ M each of dATP, dGTP and dCTP, 50 μ M of dUTP (Applied Biosystems), 200 nM each of forward and reverse primers (Integrated DNA Technologies) and 0.02 U/ μ l of HotStar *Taq* Polymerase (Qiagen). The PCR primer sequences are shown in Table 14. The PCR reaction was initiated at 95°C for 7 min, followed by denaturation at 95°C for 40 sec, annealing at 56°C for 1 min, extension at 72°C for 1 min for 50 cycles, and a final incubation at 72°C for 3 min.

Table 14. Primer sequences for reverse transcription and PCR amplification of the *SERPINB2* gene

Primer	Sequence (5' to 3')
Forward PCR primer	ACGTTGGATGTGATGCGATTTTGCAGGCAC
Reverse PCR primer	ACGTTGGATGCAGACTTCTCACCAAACAGC

[0139] **SNP detection by primer extension reaction.** The primer extension reaction was performed as described in Example 3. The PCR product was first treated with shrimp alkaline phosphatase (Sequenom, San Diego, USA). Four microliters of base extension cocktail containing 771 nM of extension primer (Integrated DNA Technologies), 1.15U of Thermosequenase (Sequenom) and 64 μ M each of ddATP, ddCTP, ddTTP and dGTP (Sequenom, San Diego, USA) were mixed with 5 μ l of water and 5 μ l of the PCR product. The thermal profile was 94°C for 2 min, followed by 94°C for 5 sec, 52°C for 5 sec, and 72°C for 5 sec for 85 cycles. Sequences of the primer extension products for each SNP allele are shown in Table 15. The molecular weights of the final extension products were detected by the MALDI-TOF mass spectrometer as described in Example 3. The ratios of the peak frequencies for the two SNP alleles in fetuses heterozygous for the SNP were determined.

Table 15. Sequences and molecular weights of the extension primer and the expected extension products for the coding SNP of *SERPINB2* gene

	Sequence	Mass (Da)
Unextended primer	TCAATGCATCCACAGGG	5179.4
Extension product		
for allele A	TCAATGCATCCACAGG G A	5476.6
Extension product		
for allele G	TCAATGCATCCACAGGG G A	5805.8

Letters in bold font indicate the dNTPs and ddNTPs residues added to the extension primer.

5 Deviation of allelic ratios of *SERPINB2* transcripts in the trisomy 18 placentas from the normal placentas

[0140] The SNP ratios of *SERPINB2* mRNA in the placentas of karyotypically normal and trisomy 18 pregnancies were compared. The ratio was calculated by dividing the relative amount of allele G (higher-mass allele) to the relative amount of allele A (lower-mass allele).

10 As shown in Figure 12, all trisomy 18 samples showed allelic ratios that deviated from the normal samples without overlapping. The allelic ratios of the trisomy 18 samples segregated into two groups. Trisomy 18 samples with an extra G allele showed allelic ratios higher than the normal range, while a sample with an extra A allele showed allelic ratio lower than the normal range.

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EXAMPLE 6: DETECTION OF TRISOMY 13 FETUS USING FETAL-EXPRESSED RNA IN PLACENTAS BY PRIMER EXTENSION AND MASS SPECTROMETRY

[0141] In order to demonstrate that additional chromosomal disorders can be detected using other genes, *collagen type IV alpha 2 (COL4A2)* (GenBank Accession number: X05610) was

20 investigated for the ability to detect trisomy 13. Primer extension reaction assays enabled determination of the SNP genotype of the placental tissue samples. The samples were processed using mass spectrometry to differentiate the different RNA-SNP alleles, and determine the relative expression levels of the RNA-SNP alleles in order to calculate the ratio of alleles. The difference in the ratios of the alleles of a *COL4A2* SNP for pregnancies with

25 and without trisomy 13 is sufficiently great that detection of trisomy 13 in the fetus is possible using placental RNA samples.

Determination of SNP ratio of *collagen type IV alpha 2* mRNA in placentas

[0142] The placental-expressed *collagen type IV alpha 2* (*COL4A2*) mRNA was chosen. The *COL4A2* gene is located on chromosome 13. A polymorphic SNP (Table 16) located within the coding region of *COL4A2* gene was identified from a public database and was targeted for assay development.

Table 16. A polymorphic SNP within the coding region of *COL4A2* gene

SNP	Sequences
COL4A2 (rs7990383) ¹	5' - GACGAAGCTATCAAAGGTCTTCCGGGACTGCCAGGACCCAAGGGCTTCGCAGGCATCAACG GGGAGCCGGGGAGGAAAGGGGACA [G/A] AGGAGACCCCGGCCAACACGGCCTCCCTGGGTTCCT AGGGCTCAAGGTGAGGAGCAATTTTCATCATGAAGCTGGCAAGACACTCTGAGGCCTCCCCA - 3'

¹ dbSNP accession number

[0143] **Sample collection and processing.** First- and second-trimester placental tissue samples were obtained from three pregnant women carrying trisomy 13 fetuses. Placental samples from seven first-trimester pregnant women carrying karyotypically normal fetuses were also collected by chorionic villus sampling (CVS). The placental samples were stored in RNeasyTM (Ambion[®], Austin, TX) immediately upon collection and kept at -80°C until RNA extraction. Total RNA was extracted with Trizol (Qiagen, Hilden, Germany) following the manufacturer's protocol. The extracted RNA was treated with DNase (Invitrogen, Carlsbad, CA, USA) to remove any contaminating DNA.

[0144] **Reverse transcription and PCR amplification.** 1.25 micrograms of the placental RNA were reverse transcribed in a reaction volume of 40 µl according to the manufacturer's instruction (ThermoScript, Invitrogen, Carlsbad, CA, USA). The reverse transcription was performed using the reverse PCR primer with sequence shown in Table 17.

[0145] For the PCR amplification, 40 µl of the placental cDNA was used in a total volume of 80 µl. Each reaction contained 0.6X HotStar *Taq* PCR buffer with 0.9 mM MgCl₂ (Qiagen), 25 µM each of dATP, dGTP and dCTP, 50 µM of dUTP (Applied Biosystems), 200 nM each of forward and reverse primers (Integrated DNA Technologies) and 0.02 U/µl of HotStar *Taq* Polymerase (Qiagen). The PCR primer sequences are shown in Table 17. The PCR reaction was initiated at 95°C for 7 min, followed by denaturation at 95°C for 40 sec, annealing at 56°C for 1 min, extension at 72°C for 1 min for 50 cycles, and a final incubation at 72°C for 3 min.

Table 17. Primer sequences for reverse transcription and PCR amplification of the *COL4A2* gene

Primer	Sequence (5' to 3')
Forward PCR primer	ACGTTGGATGAAGGGCTTCGCAGGCATCAA
Reverse PCR primer	ACGTTGGATGACCAATGTTGCCAGGCACTC

[0146] *SNP detection and allelic ratio quantification by primer extension reaction.* The primer extension reaction was performed as was described in Example 3. The PCR product was first treated with shrimp alkaline phosphatase (Sequenom, San Diego, USA). Four microliters of base extension cocktail containing 771 nM of extension primer (Integrated DNA Technologies), 1.15U of Thermosequenase (Sequenom) and 64 μ M each of ddATP, ddCTP, ddGTP and dTTP (Sequenom, San Diego, USA) were mixed with 5 μ l of water and 5 μ l of the PCR product. The thermal profile was 94°C for 2 min, followed by 94°C for 5 sec, 52°C for 5 sec, and 72 °C for 5 sec for 100 cycles. Sequences of the primer extended products for each SNP allele are shown in Table 18. The molecular weights of the final extension products were detected by MALDI-TOF mass spectrometry as was described in Example 3. The peak frequency ratios for the two SNP alleles in fetuses heterozygous for the SNP were determined.

Table 18. Sequences and molecular weights of the extension primer and the expected extension products for the coding SNP of *COL4A2* gene

	Sequence	Mass (Da)
Unextended primer	GTTGGCCGGGGTCTCCT	5209.4
Extension product for allele G	GTTGGCCGGGGTCTCCT C	5482.6
Extension product for allele A	GTTGGCCGGGGTCTCCT TTG	6131.0

The extension primer anneals to the reverse strand of the sequence.

Letters in bold font indicate the dNTPs and ddNTPs residues added to the extension primer.

Deviation of allelic ratios of *COL4A2* transcripts in the trisomy 13 placentas from the normal placentas

[0147] The allelic ratios of *COL4A2* mRNA in the placentas of karyotypically normal and trisomy 13 pregnancies were compared. The ratio was calculated by dividing the relative amount of allele A (higher-mass allele) to the relative amount of allele G (lower-mass allele). As shown in Figure 13, all trisomy 13 placentas showed allelic ratios that deviated from the normal samples without overlapping. The allelic ratios of the trisomy 13 samples segregated

into two groups. The trisomy sample with an extra A allele showed allelic ratio higher than the normal range, while samples with an extra G allele showed allelic ratios lower than the normal range.

- 5 [0148] Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, one of skill in the art will appreciate that certain changes and modifications may be practiced thereto.

Table 19. Genomic sequence of *PLAC4* gene locus.

This *PLAC4* gene locus spans the nucleotide coordinates 41469028-41480585 on chromosome 21 based on the Human May 2004 (hg17) assembly at the UCSC Genome

Browser (a website having a database of these gene sequences and nucleic acid sequences for genomes offered at the time of preparation of this application based in the U.S.).

chr21: 41469028-41480585

Base position¹

41469028	TGGGATGTTTTTCAGATTTTTATTATATGGCAATCATATACCTGCACCTAGAAATATACAA
41469088	CCTCGGCGCTGCCATTGCAGGAAGACAAAGAGACTGTCTAGAGAGTAACGTGGCGATGCC
41469148	CTGGGCAGCTCCATCATTCCAGGGTACCATAAAGGGAAGTGGGAATGCACACTCCATCTG
41469208	TTTCACGCTAGGCTGAAAGTGGCAGGGGGAGAATTTACACCTGGCCTGCAAAAGGCAGCC
41469268	TTGTGTTCCCACTTCAGAGCCCCAACTTCTCCAAAGCCAGTGCTTGGGAAATGGCCTTGT
41469328	TGGCAGCAGGCAGGAGACTGGTGCAGTGTGCCAGCTGTGCAAACCCCCACCAGACTGGTG
41469388	CAGTGTGCCAGCTGTGCAAACCCCCACCAGCAGCAGCCCCACCAGCAGCCAAAGAAAACA
41469448	ATTTCTAAAGCAGCCCCCAGGATTTTCATGCCACGGGGGTGGTGAGGGGTAGGAGGAGGTG
41469508	GAAAGCATTGAATCAGAAAGTCTTCCAGGTACCAGCAATGCCAGGGTTAATATGGTCTGG
41469568	CTTGCTTTTCAGGTGAGCACCTGTAGCTGGCAGGAAAGTGGCTTCTTGGTGGAGGTGGGC
41469628	ATGAATTCAGAAGCCTTGGAGAGACATCCAGAGCCCTTCCCCTACATCGTGTCTGCCTC
41469688	CTGGGGAAATTCCTAAGCTTTTTTTGAATCAGAAAAGCCACTGACAAGCAGACAGAATTG
41469748	TGTGGCTTGCGCAGTGATTGGAGAGCTAGGTGCTTTGGGTACTAGTCCCAGAGCTGCTAC
41469808	TTGTCGAATGTTGGGTATTGGAGAGAAGTCATTTGAGCTGTCTGAGCCTCTGTTTCTCA
41469868	TCTGTAAATCAGGGAATTTGAACAAGTGACCTCAGATTCTTCTAGAAGCTCTAACAGTC
41469928	AATGATATCATCTATTTTCATTTGAGAGAATCTCCATAGCTCTAATTTTTTGGCCCCAGAC
41469988	CAATCTGCTTCAGCTTTGTGTGGGTGCAACACCTGGGGTCCTGTAAATGCAGAATCGG
41470048	ATTCAGTGACCGAGAGCAGAGCTGAGGGTGGCTGCTGCCAGCTCACAGGTCACACTTGGA
41470108	GTCGTTGCTTCTTTCCCCAAACCAGCTGCCGATGGTTCTTGGAAGAGCAGGAATACCTT
41470168	GCAGAAGGGCCTAGAAATAGAGATTCCCATATGCAGTCCAGACTTATTGGGTGAGCCTGT
41470228	CTGCAC'TAGGACCTGAGAATCTGCATTTTATTATATCCCTTGAGTCCCCTTAAGCAGCC
41470288	ACGCTGGCCCCAGCCATTGGACTCTATTTGGAGGCCACTGCAGAGGCCAGATGCCCTCT
41470348	GCTCGGCGGTTTCTGTGCAGAAAGGCTGTGTTCTTTCTTCTTCTAATACTCTTCTTA
41470408	GGTTAAGTGTTTCTTCTTCTTGGCCATCTAGAAAATCTCCACCTCAAGCAGGTGCTGTT
41470468	CCAAAACTCCTCGTGTGGGCAAGTGGAACACGCAGTTTTTTCTAGGGGTCTCATTCT
41470528	TGAGGCGGTGCTGCTGAAAATCTTGGTGCTGAACGTGTGTTTTTTGAGATTTCCAGTCTAT
41470588	CACAGGGCCACAAGGTGTAAATATCAAGAAAAATGAATTACTAGAAAGGCAAAGTGAAAA
41470648	AGACATACAAAATACAAGTGTCAATCTTTTATTCTTAGTTTGGACAGATAGTCCATTCT
41470708	<u>TCTACACTGGTCCCACGGTTTCTGAAGCTTCTGTAAATGATCAGTGGTTACCTTGTGTC</u>
41470768	<u>CAAGTAACTGCAAAATCCCTCCTCTACAAAGTGTGCTTGCTCCAAGGCAGTGCAAACTAGA</u>
41470828	<u>AGTTGTTACAAATGGTTCCAGTAACAAATTTGTTTGGCGGCCTTTTCCCATGAATGAGAC</u>
41470888	<u>AGTGGTTATTTTTGCTAAAGCAGAAAGGAAATGTGATACTATTGGGCTGTGTTTTGCCCT</u>
41470948	<u>CTGGAGTAATCCTGCTTGGGGAAAATGGAGGCTTGTTCAGAAATGCAGAAATCCCTGTTA</u>
41471008	<u>AATTAGGCAGTCTTGGGCTGGAGGACGTGTGCCTGCCCTCCCAGTGCTCACAACCTCACA</u>
41471068	<u>TCTCTAAAGGCCACTCAGGTGGGACACCATCAAGACATTGAAAATCGACAGAAGGAAGGC</u>
41471128	<u>AGGAAGGGGAGAGGATCGAACCTGTCAAAATAGATATTCAGAAAATCTGTGCTCTAAAAT</u>
41471188	<u>AAGGCAGCCCTTCCCTCACAGCACACTTATTCCTAATTTCAACAGGACTCCTAGTCTTGC</u>
41471248	<u>CCCACAGCGTCACAGCCTACAGCAAATTAGAACTGGGGTGGGGGGCGGATATTATTCCA</u>
41471308	<u>CCAGTAATACCTTGGGACGGGGCACACAAGATGTTTGCCCTCCTACCTCTCTGTACCT</u>
41471368	<u>TCCCAAGAAAGGGTCAAGATGAAACAGTGTGCGTTTATGGTATTGCGAGAGTTAAGTGAG</u>
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 41480548 GATCAGGGCTGCTCCATAGGCTGTGTGCCCTGAGTAGC

Bold fonts: sequence known or predicted to be the transcribed regions of the *PLAC4* gene.

Underlined fonts: reference sequence corresponding to the *PLAC4* mRNA from NCBI database (NM_182832)

¹ Nucleotide coordinates on chromosome 21 based on the Human May 2004 (hg17)

Assembly at the UCSC Genome Browser (a website having a database of these gene sequences and nucleic acid sequences for genomes offered at the time of preparation of this application based in the U.S.).

DEMANDES OU BREVETS VOLUMINEUX

**LA PRÉSENTE PARTIE DE CETTE DEMANDE OU CE BREVETS
COMPREND PLUS D'UN TOME.**

CECI EST LE TOME __1__ DE __2__

NOTE: Pour les tomes additionels, veuillez contacter le Bureau Canadien des Brevets.

JUMBO APPLICATIONS / PATENTS

**THIS SECTION OF THE APPLICATION / PATENT CONTAINS MORE
THAN ONE VOLUME.**

THIS IS VOLUME __1__ OF __2__

NOTE: For additional volumes please contact the Canadian Patent Office.

CLAIMS:

1. A method for detecting the presence of a chromosomal disorder in the fetus of a pregnant woman, comprising the steps of:
 - (a) discriminating alleles from fetal RNA transcribed from at least one genetic locus from at least one chromosome of concern in an RNA-containing biological sample from the pregnant woman, wherein the RNA-containing biological sample contains fetal RNA and said genetic locus is selected from the group consisting of *collagen V1 alpha 1 (COL6A1)*, *collagen V1 alpha 2 (COL6A2)*, *placenta-specific 4 (PLAC4)*, *serpin peptidase inhibitor clade B (ovalbumin) member 2 (SERPINB2)* and *collagen type IV alpha 2 (COL4A2)*;
 - (b) determining a ratio of the alleles of the RNA transcripts; and
 - (c) comparing the ratio from step (b) to a standard control representing a ratio of alleles from comparable biological samples obtained from pregnant women each carrying a chromosomally normal fetus, wherein an increase or decrease in the determined ratio of step (b) to the standard control indicates an increased risk of having a fetus with a chromosomal disorder.
2. The method of claim 1, in which the chromosomal disorder is a member selected from the group consisting of trisomy 21, trisomy 18 and trisomy 13.
3. The method of claim 2, in which the chromosomal disorder is trisomy 21.
4. The method of claim 1, in which the chromosomal disorder is on the X chromosome or the Y chromosome.
5. The method of claim 1, in which the biological sample in step (a) is a member selected from the group consisting of maternal blood, maternal plasma or serum, amniotic fluid, a chorionic villus sample, biopsy material from a pre-implantation embryo, fetal nucleated cells or fetal cellular remnants isolated from maternal blood, maternal urine, maternal saliva, washings of the female reproductive tract and a sample obtained by celocentesis.
6. The method of claim 1, in which the biological sample in step (a) is maternal blood.

7. The method of claim 1, in which the biological sample in step (a) contains cellular elements or cellular remnants in maternal blood.

8. The method of claim 1, in which the fetal RNA in step (a) is derived from the placenta.

9. The method of claim 1, in which step (a) is performed using reverse transcriptase polymerase chain reaction (RT-PCR).

10. The method of claim 1, in which step (a) and/or step (b) is performed using a member selected from the group consisting of a primer extension reaction, mass spectrometry, hybridization using at least one probe, hybridization using at least one fluorescently labeled probe, direct sequencing, cloning and sequencing, and electrophoresis.

11. The method of claim 1, in which the alleles in steps (a), (b) and (c) are differentiated by sequence variation.

12. The method of claim 11, in which the sequence variation is a single nucleotide polymorphism (SNP).

13. The method of claim 11, in which the sequence variation is an insertion and/or deletion polymorphism.

14. The method of claim 11, in which the sequence variation is a simple tandem repeat polymorphism.

15. The method of claim 1, in which the RNA is transcribed from a member selected from the group consisting of chromosome 21, chromosome 18, chromosome 13, chromosome X, and chromosome Y.

16. The method of claim 15, in which the RNA is transcribed from chromosome 21.

17. The method of claim 1, in which the RNA is mRNA.
18. The method of claim 1, wherein the RNA contains at least one single nucleotide polymorphism of the genetic locus.
19. The method of claim 18, wherein the RNA is transcribed from at least one genetic locus selected from the group consisting of *collagen VI alpha 1 (COL6A1)* and *collagen VI alpha 2 (COL6A2)*.
20. The method of claim 19, wherein the SNP in the RNA transcribed from the genetic locus of the *COL6A1* is rs1053312 for ^{Arg}850_{His} or rs1053320 for ^{Ser}932_{Ser}.
21. The method of claim 19, wherein the SNP in the RNA transcribed from the genetic locus of the *COL6A2* is rs2839114 for ^{Val}728_{Val}.
22. The method of claim 1, wherein the RNA is transcribed from the genetic locus for *placenta-specific 4 (PLAC4)*.
23. The method of claim 22, wherein the RNA is a variant transcribed from the *PLAC4* locus, selected from the group consisting of AF269287, AK027868, AK092431, BC093685, BC101615, BC101617, L13197, NM_182832 and LOC191585.
24. The method of claim 22, wherein the RNA transcribed from the genetic locus of the *PLAC4* gene contains a single nucleotide polymorphism or an insertion-deletion polymorphism selected from the group consisting of *rs3804026*, *rs4818219*, *rs7844*, *rs9015*, *rs13643*, *rs9305729*, *rs9305730*, *rs5019195*, *rs5019194*, *rs5844069*, *rs1049904*, *rs16998089*, *rs12482116*, *rs11909439*, *rs7278659*, *rs12106409*, *rs12106395*, *rs12106401*, *rs12106434*, *rs2183584*, *rs3949725*, *rs8130833*, *rs10222145*, *rs9981478*, *rs8130833*, *rs9977003*, *PLAC4-41471145* and *PLAC4-41476236*.
25. The method of claim 1, wherein the woman is during the first trimester of gestation.

26. The method of claim 1, wherein the woman is during the second trimester of gestation.

27. The method of claim 1, wherein the comparison in step (c) shows an increased risk of the fetus having a chromosomal disorder if the ratio in step (b) is higher or lower by 1 standard deviation from the standard control.

28. The method of claim 1, wherein the comparison in step (c) shows an increased risk of the fetus having a chromosomal disorder if the ratio in step (b) is higher or lower by 2 standard deviations from the standard control.

29. The method of claim 1, wherein the comparison in step (c) shows an increased risk of the fetus having a chromosomal disorder if the ratio in step (b) is higher or lower by 3 standard deviations from the standard control.

30. A kit for detecting the presence of a fetus with a chromosomal disorder in a pregnant woman, the kit comprising:

(a) primers for amplifying the alleles from fetal RNA transcribed from at least one genetic locus from at least one chromosome of concern in an RNA-containing biological sample said genetic locus is selected from the group consisting of *collagen V1 alpha 1* (*COL6A1*), *collagen V1 alpha 2* (*COL6A2*), *placenta-specific 4* (*PLAC4*), *serpin peptidase inhibitor clade B (ovalbumin) member 2* (*SERPINB2*) and *collagen type IV alpha 2* (*COL4A2*); and

(b) a standard control of DNA or RNA representing a ratio of alleles from comparable biological samples obtained from pregnant women each carrying a chromosomally normal fetus.

31. The kit of claim 30, further comprising:

(c) hybridization probes for discriminating between the different alleles of each RNA species.

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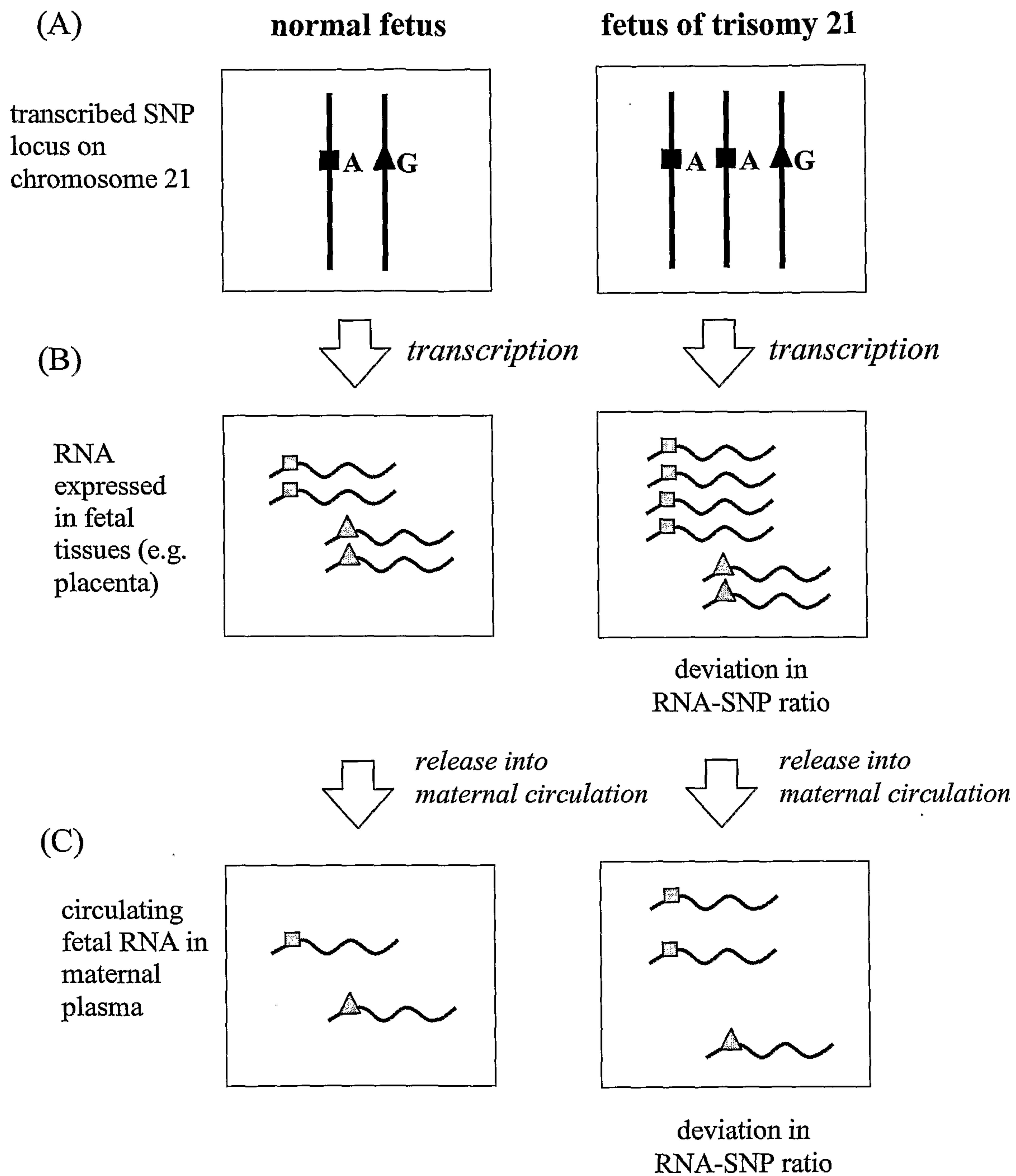
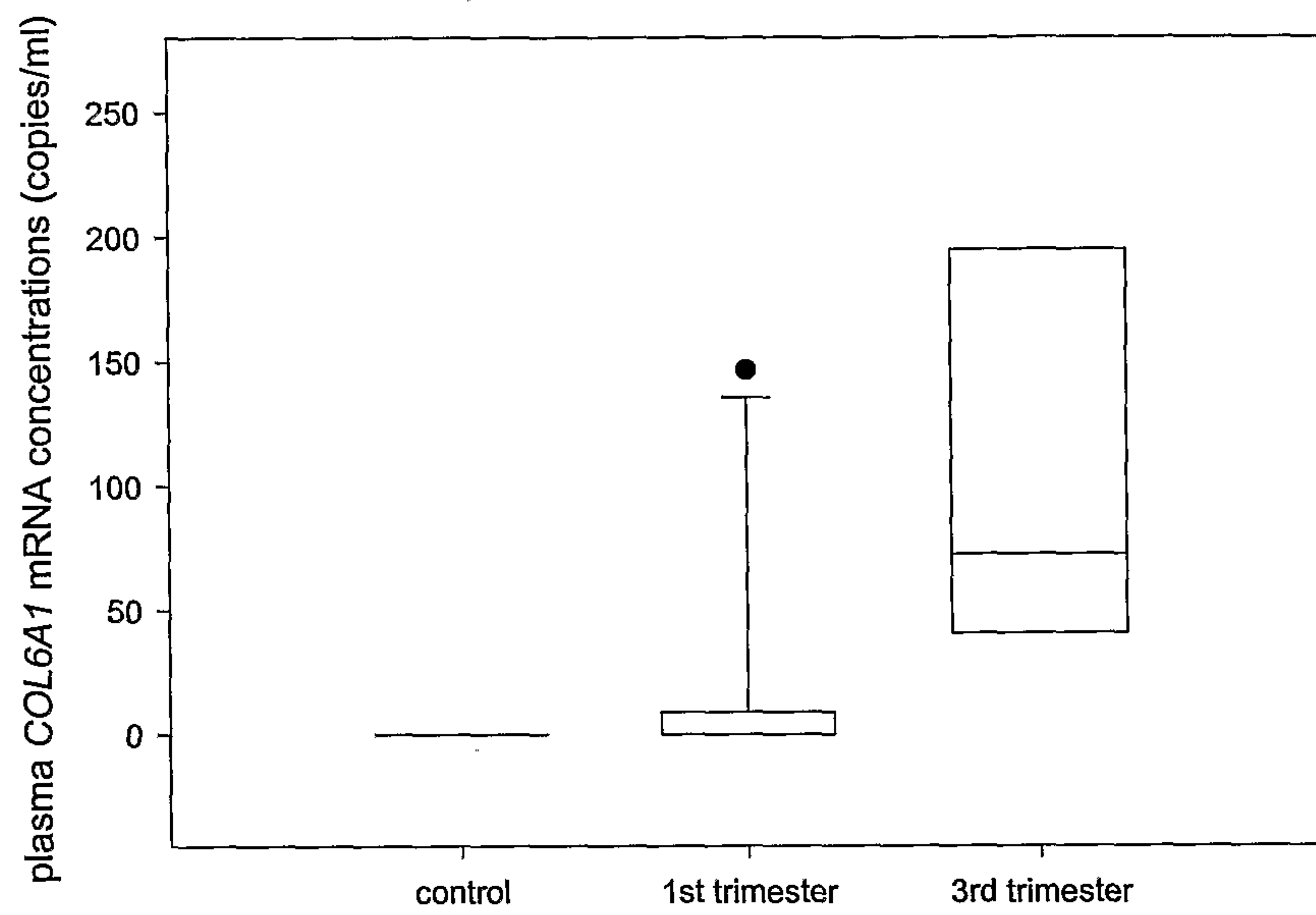


Figure 1

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(A)



(B)

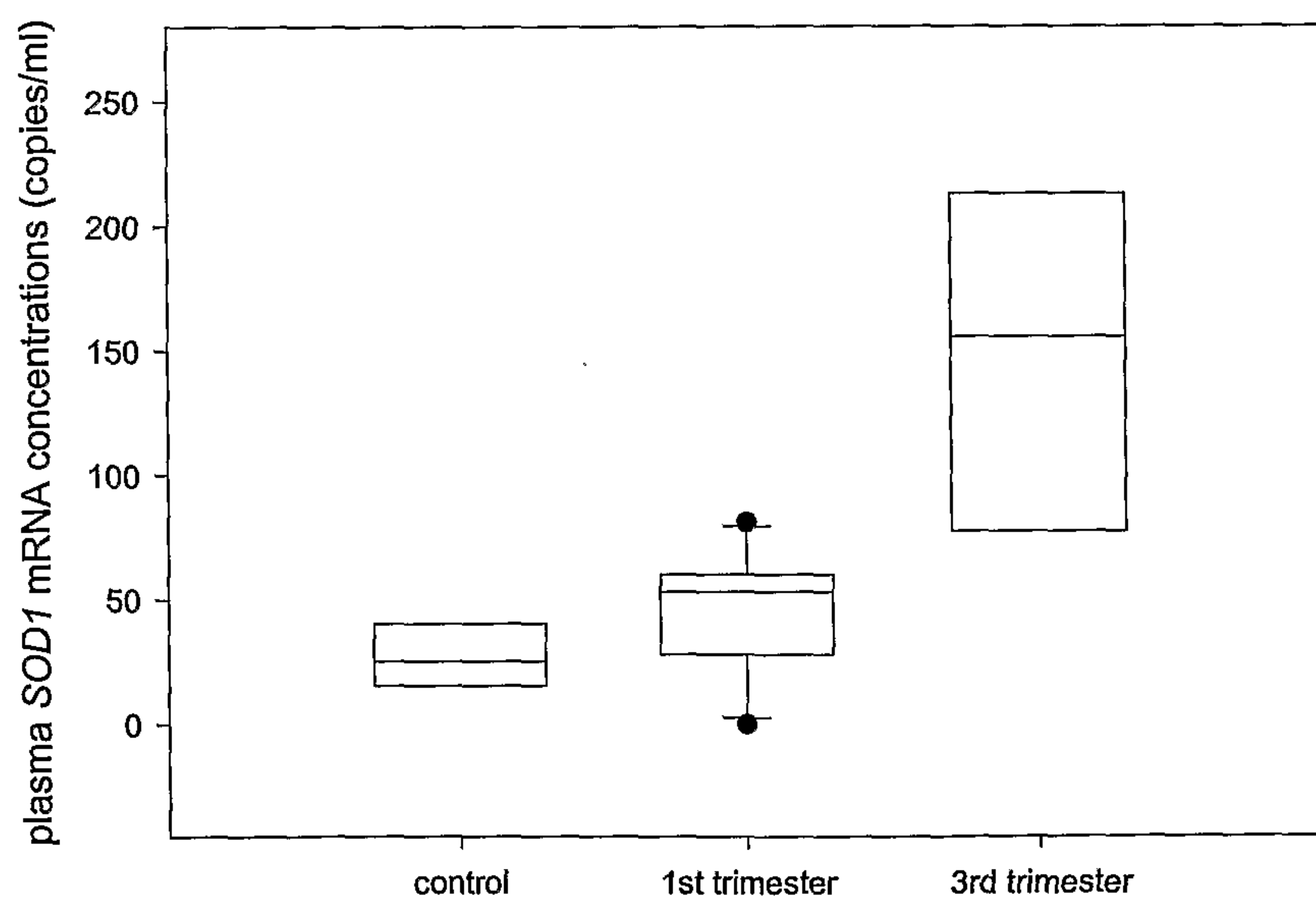
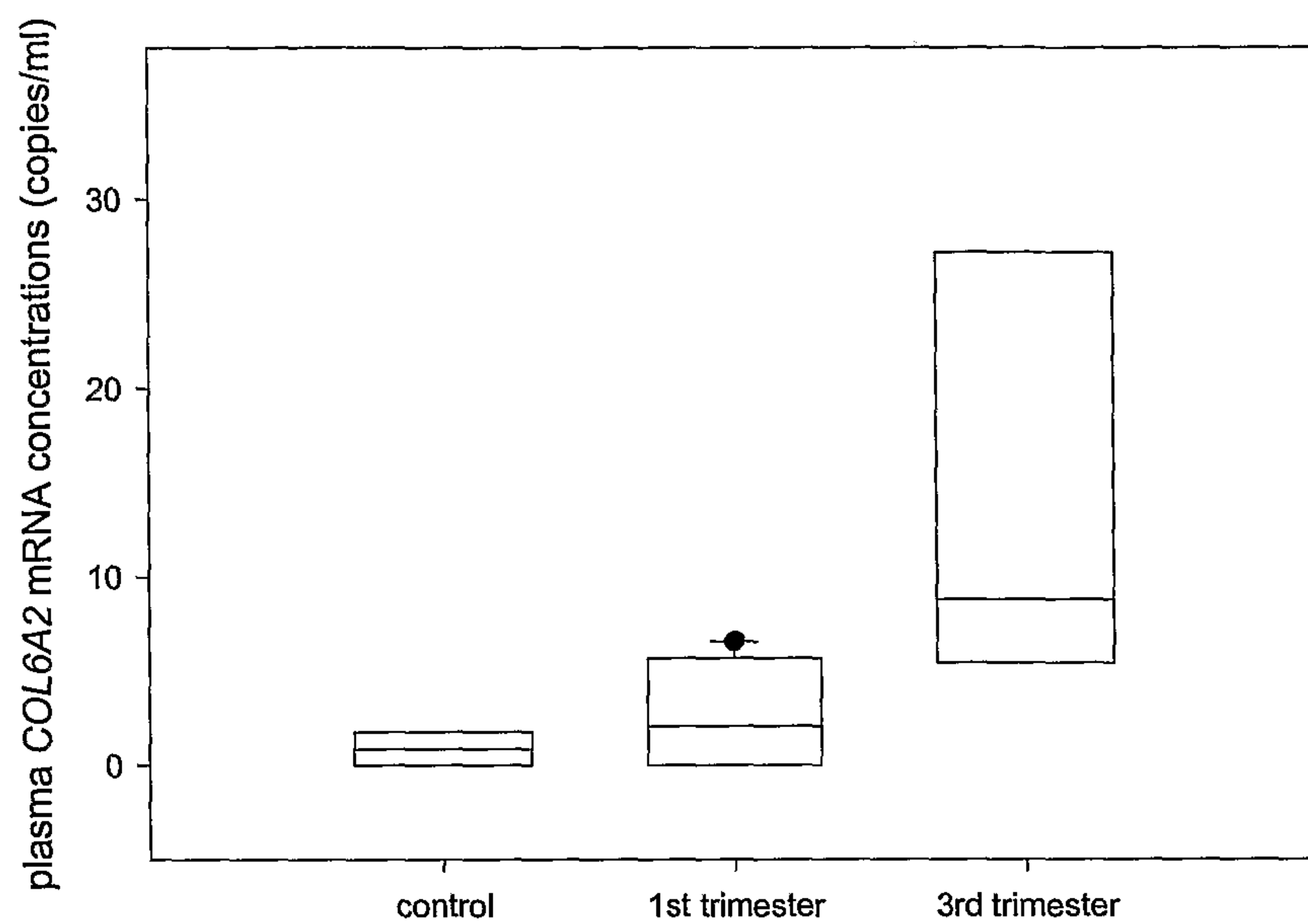


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(C)



(D)

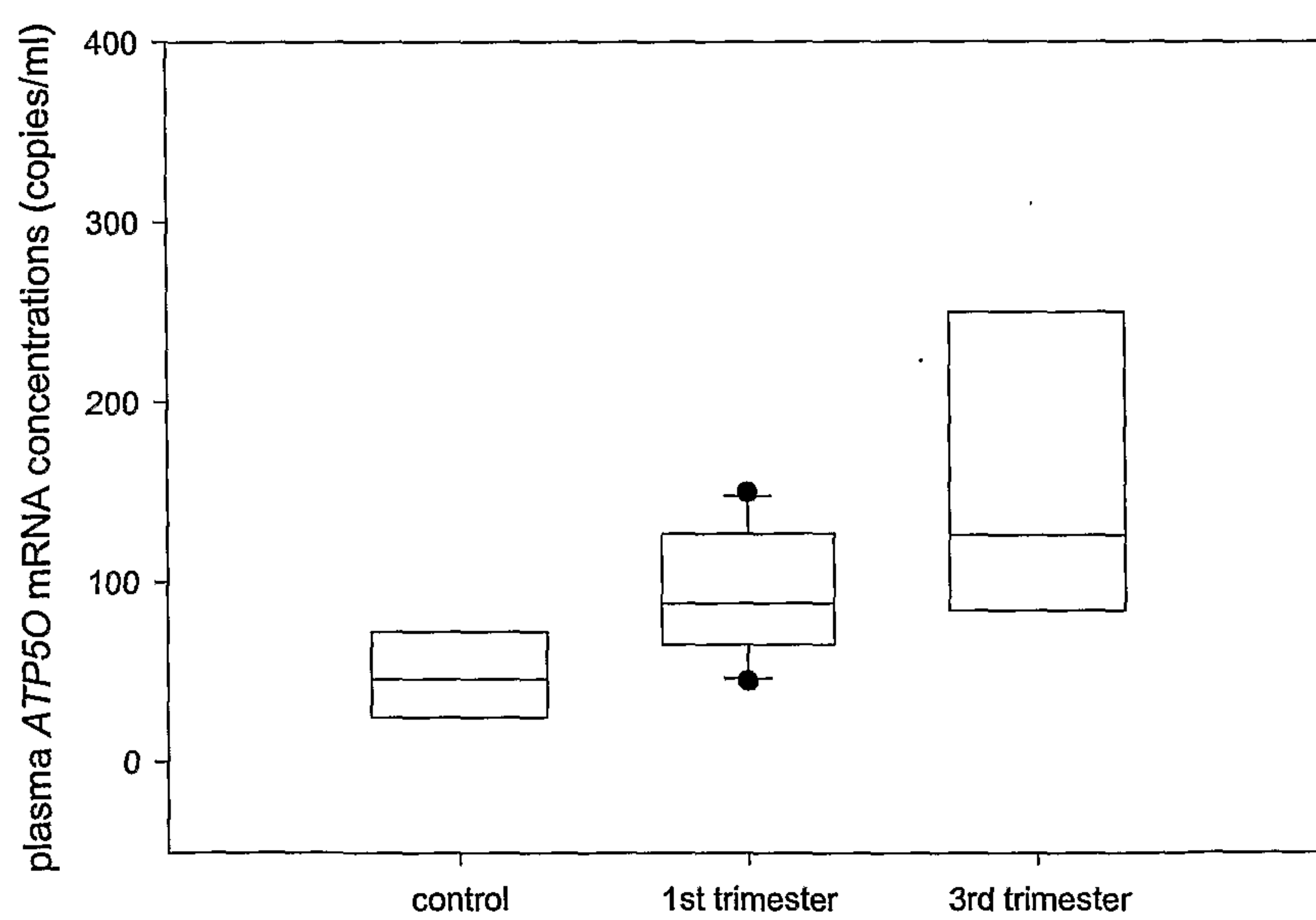
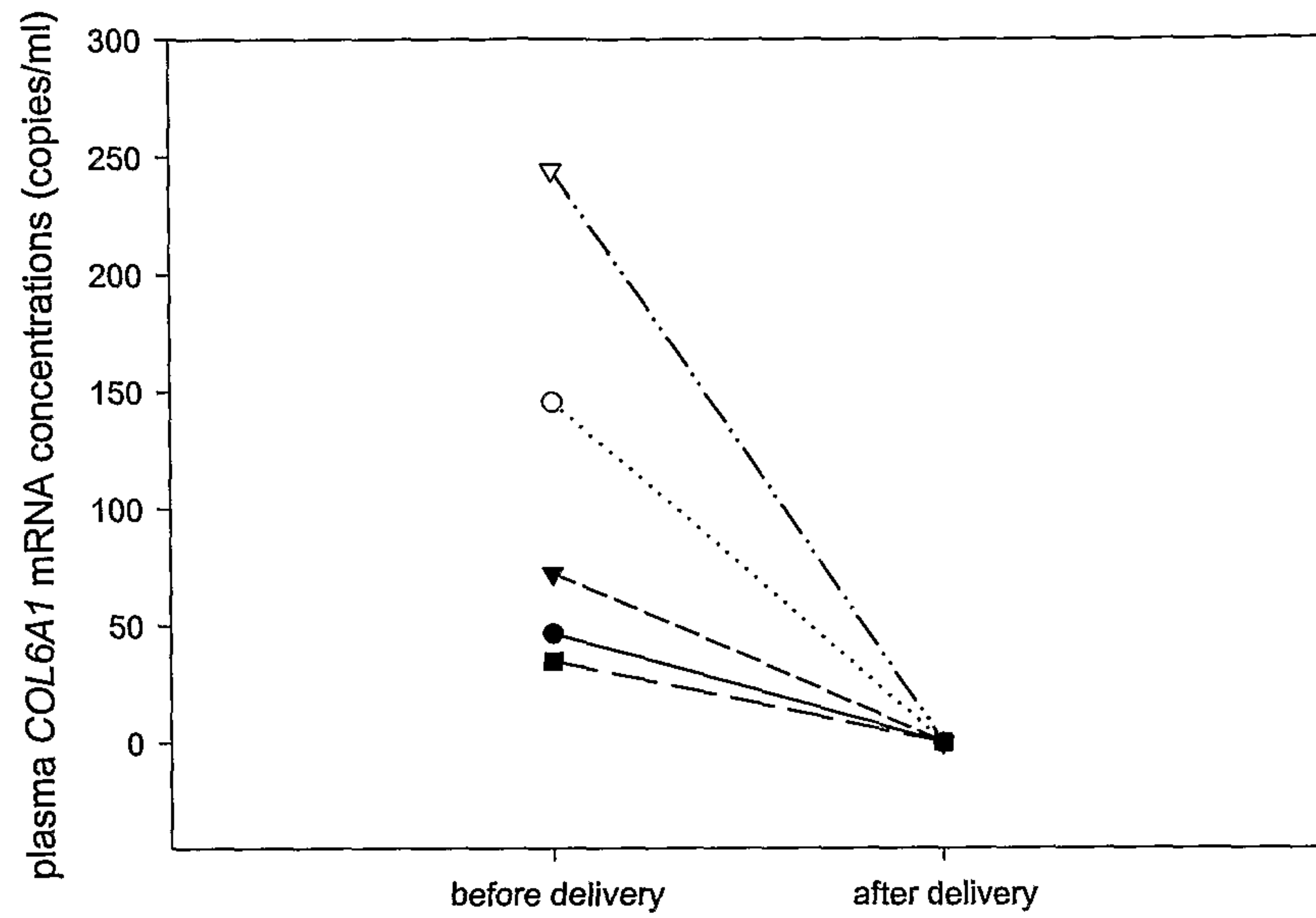


Figure 2 2/2

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(A)



(B)

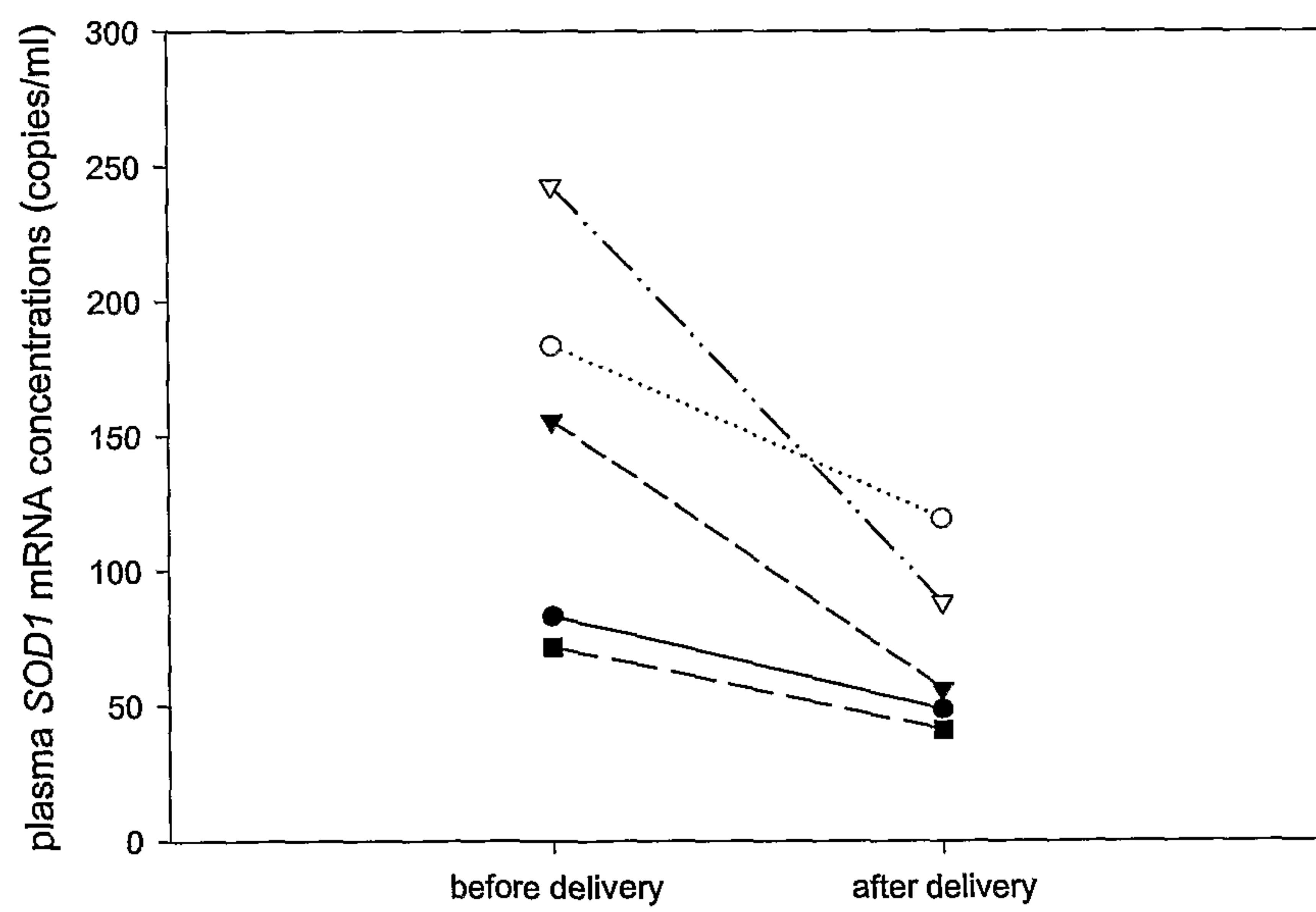
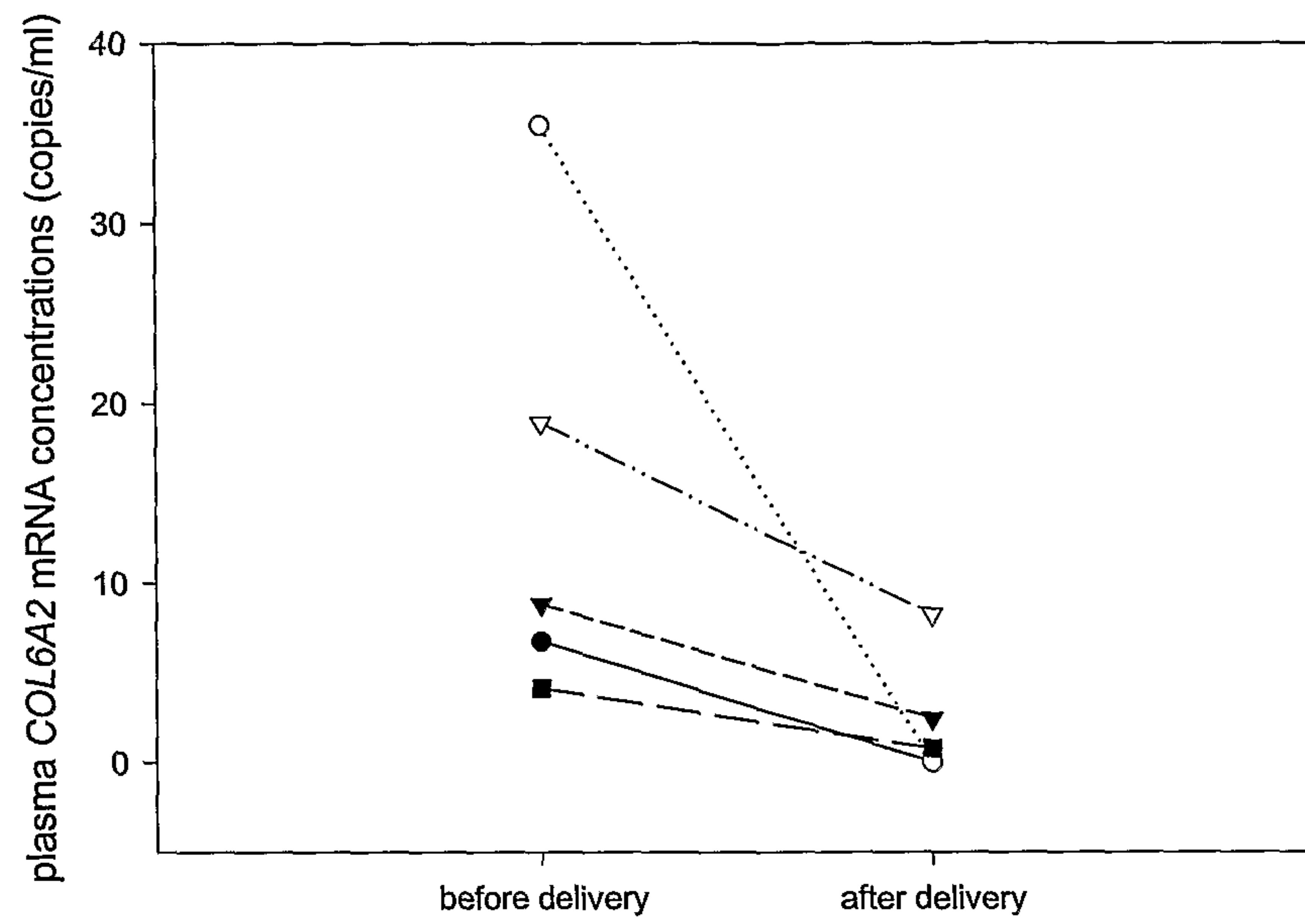


Figure 3 1/2

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(C)



(D)

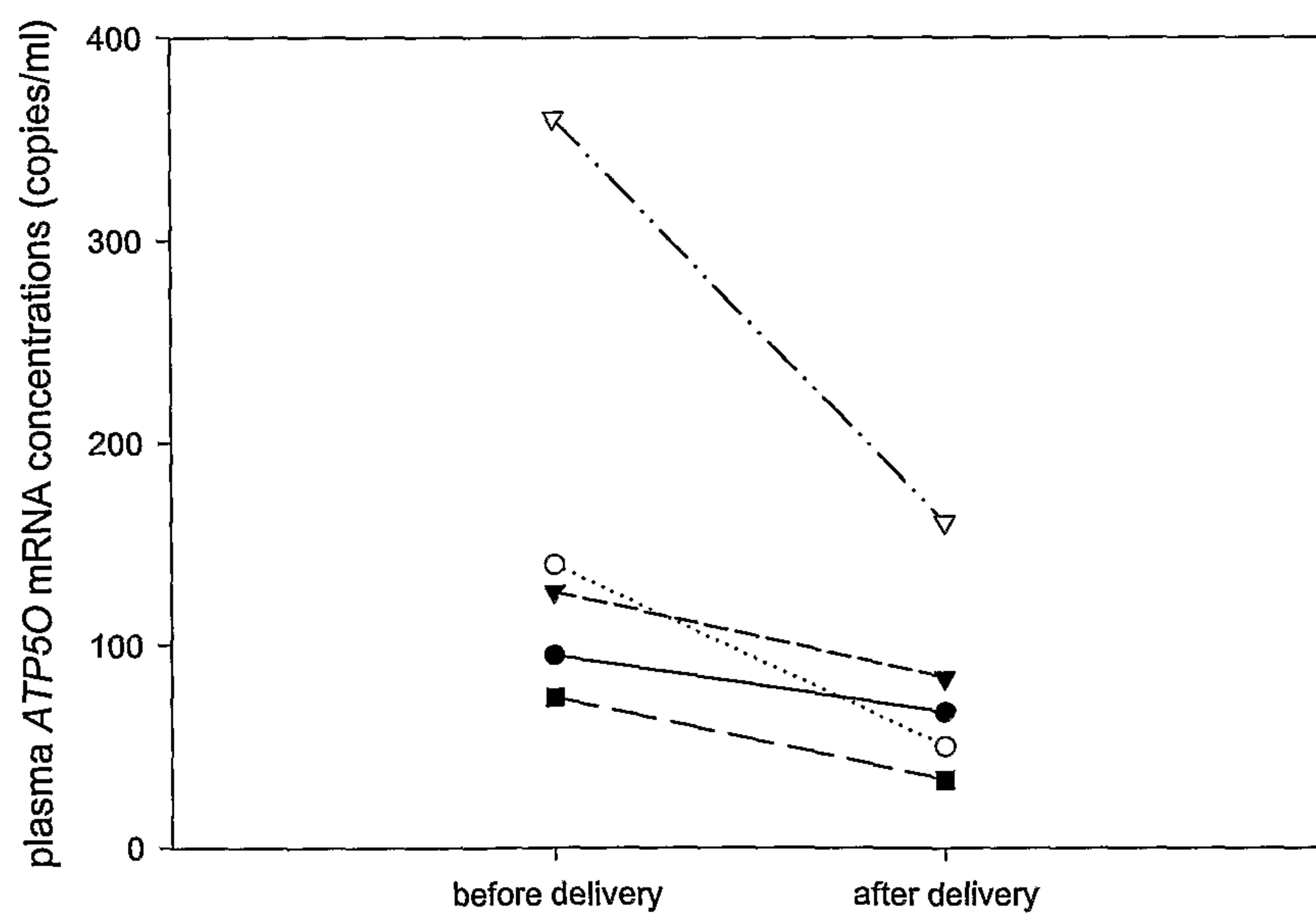


Figure 3

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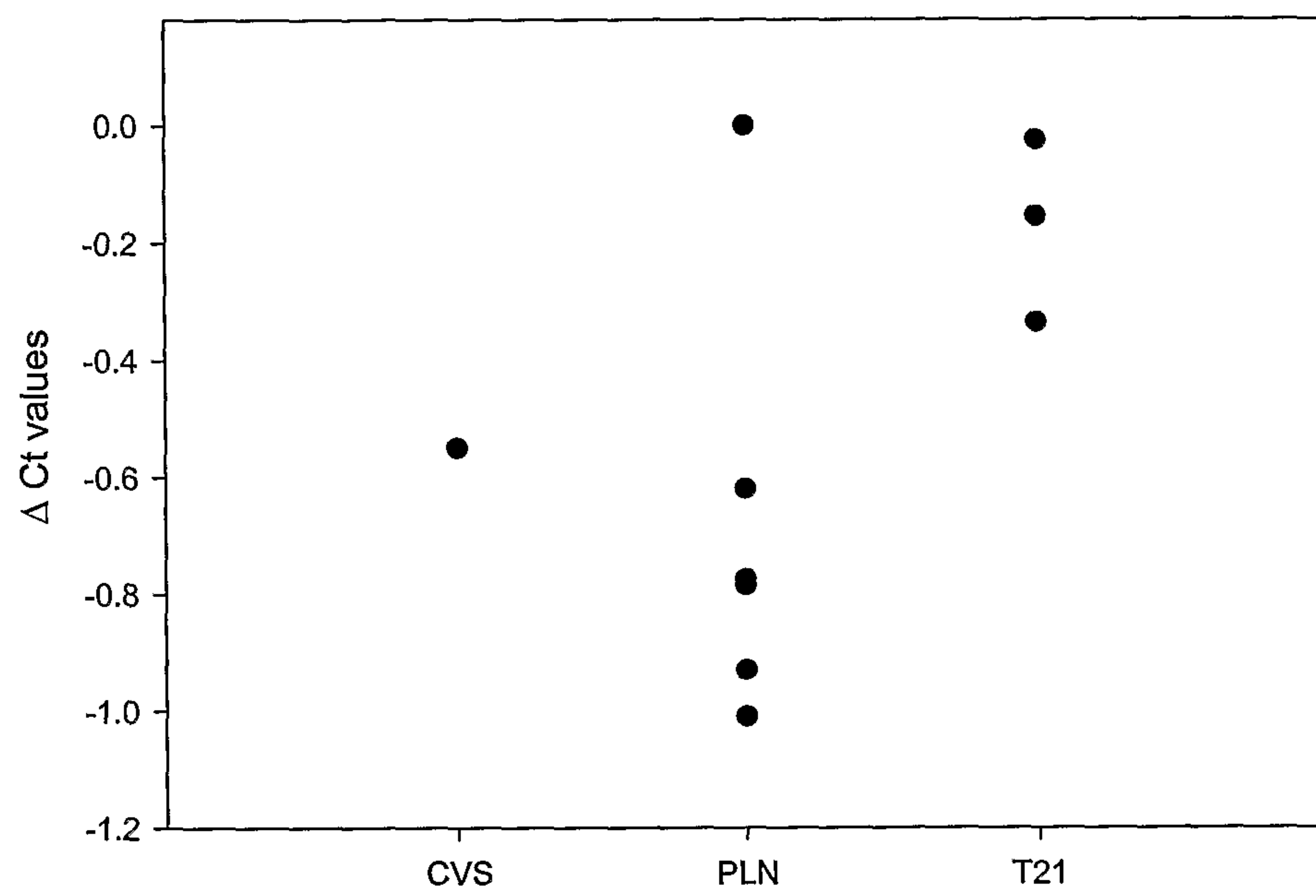
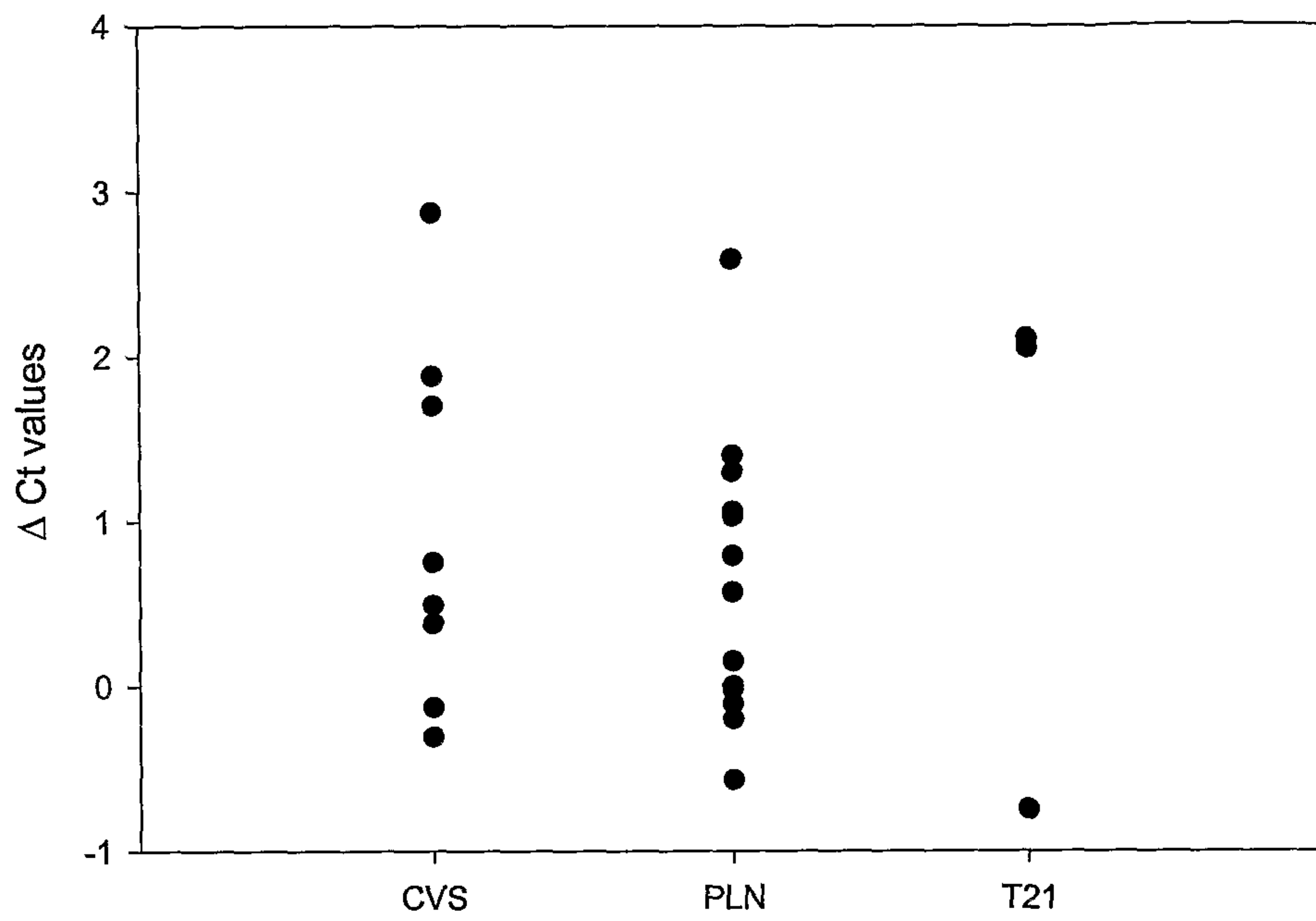


Figure 4

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(A)



(B)

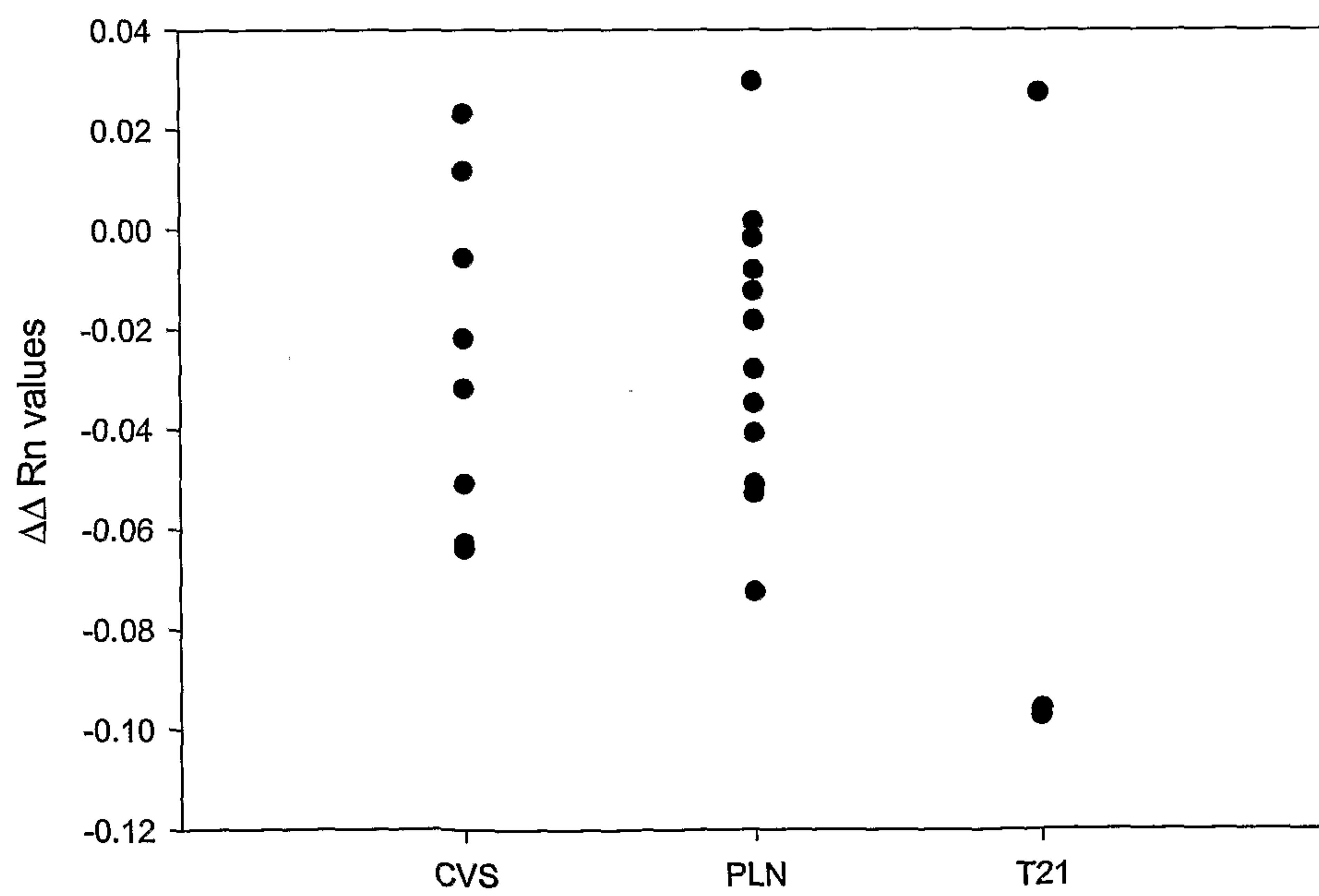
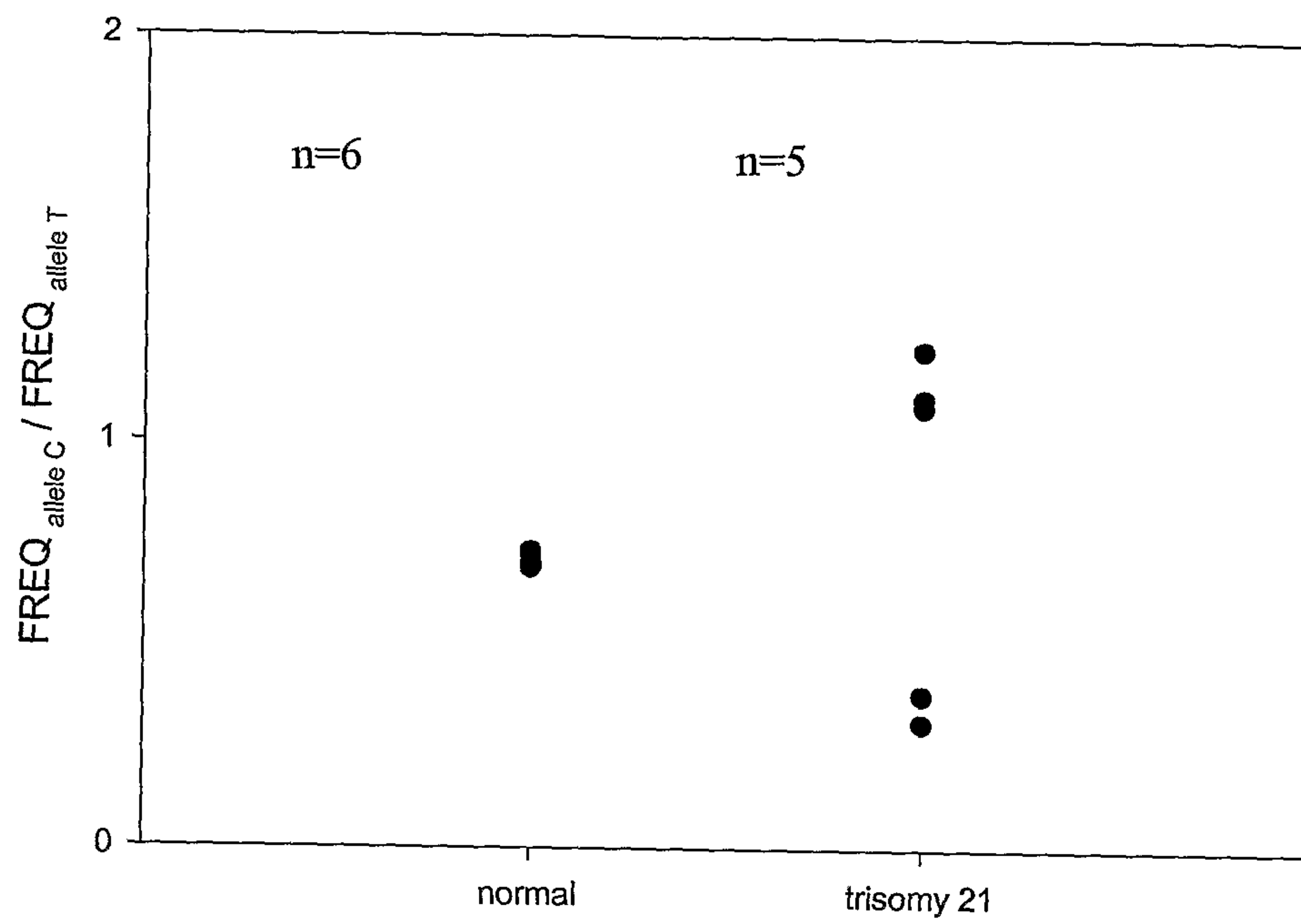


Figure 5

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(A)



(B)

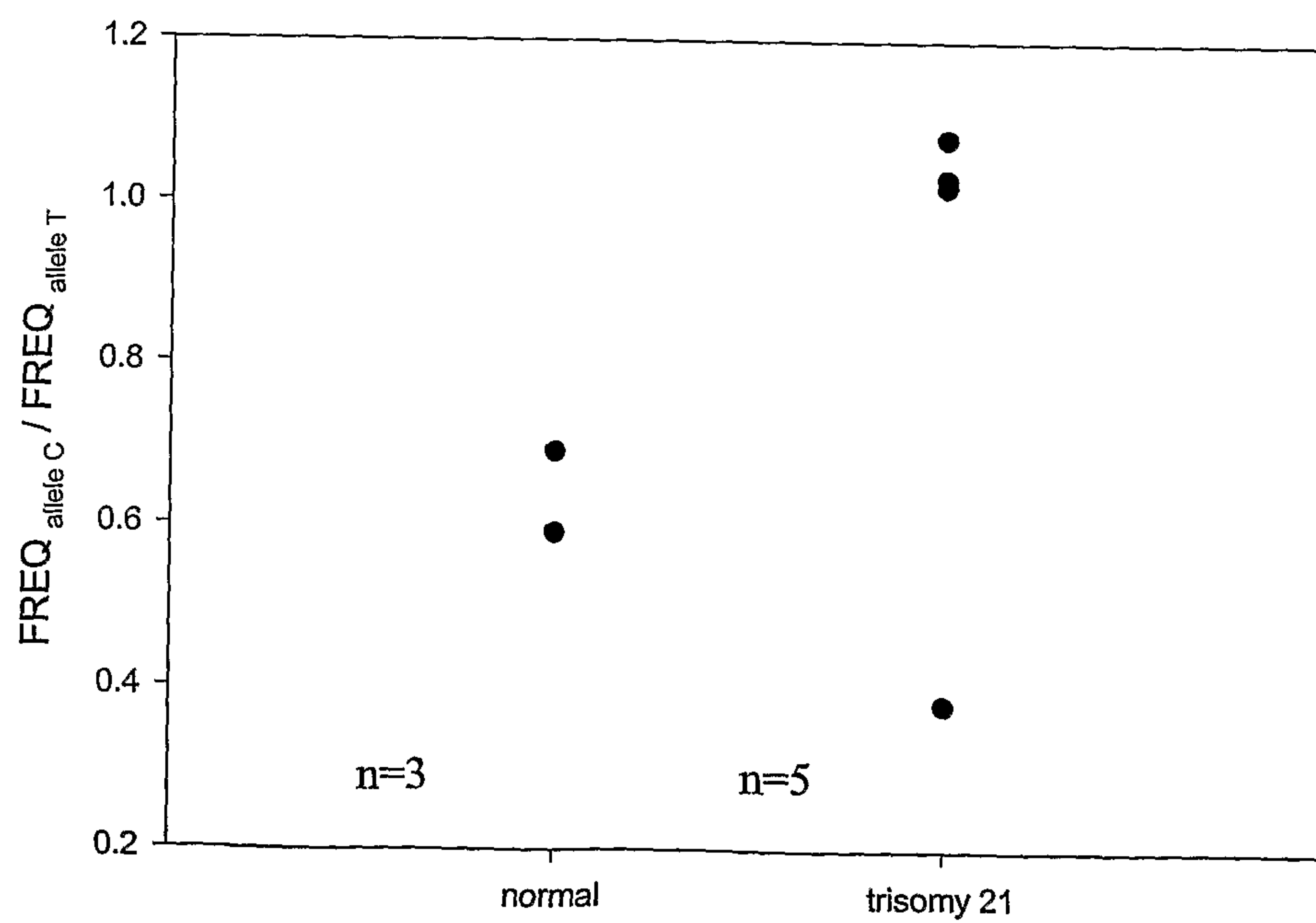
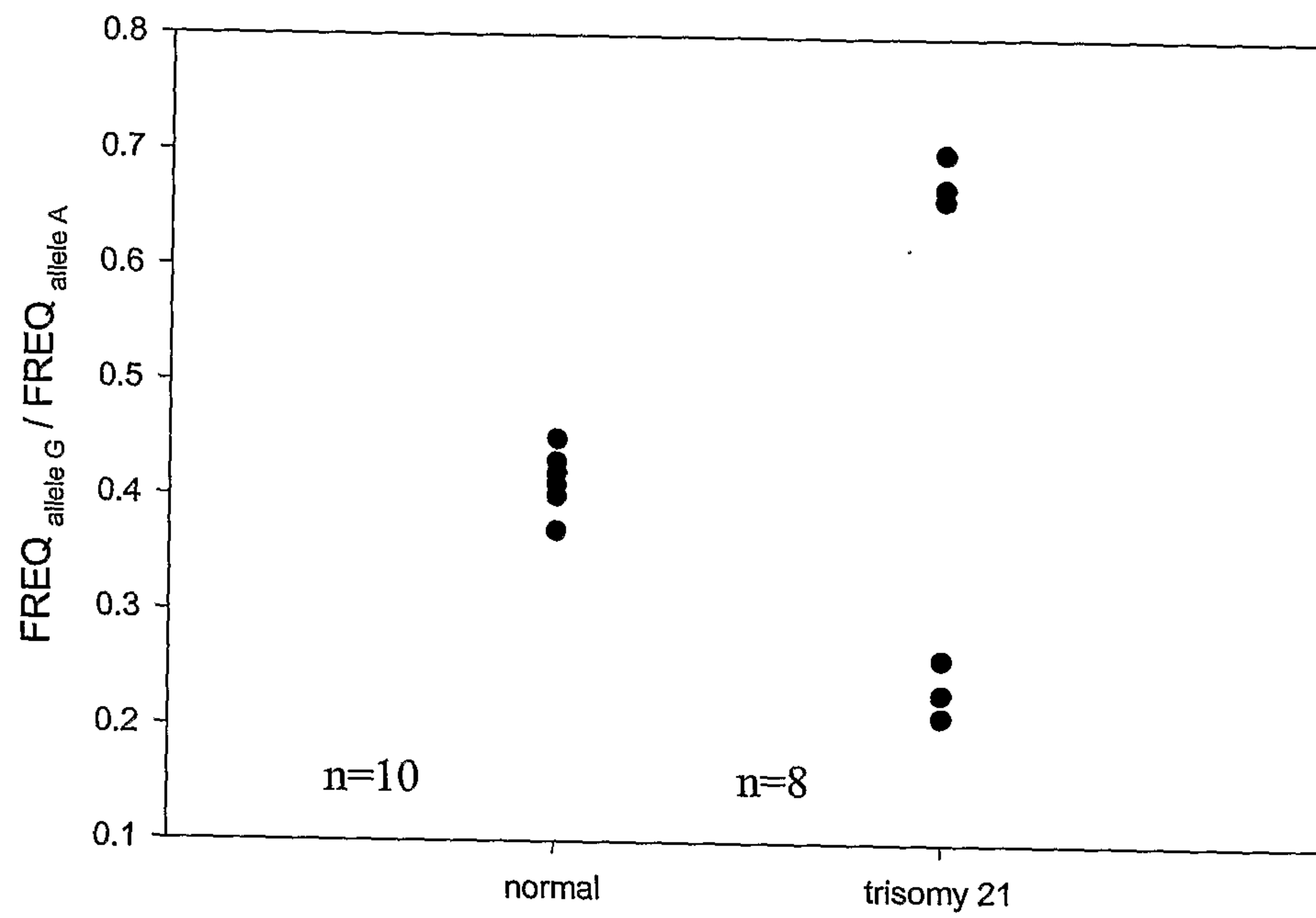


Figure 6

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(A)



(B)

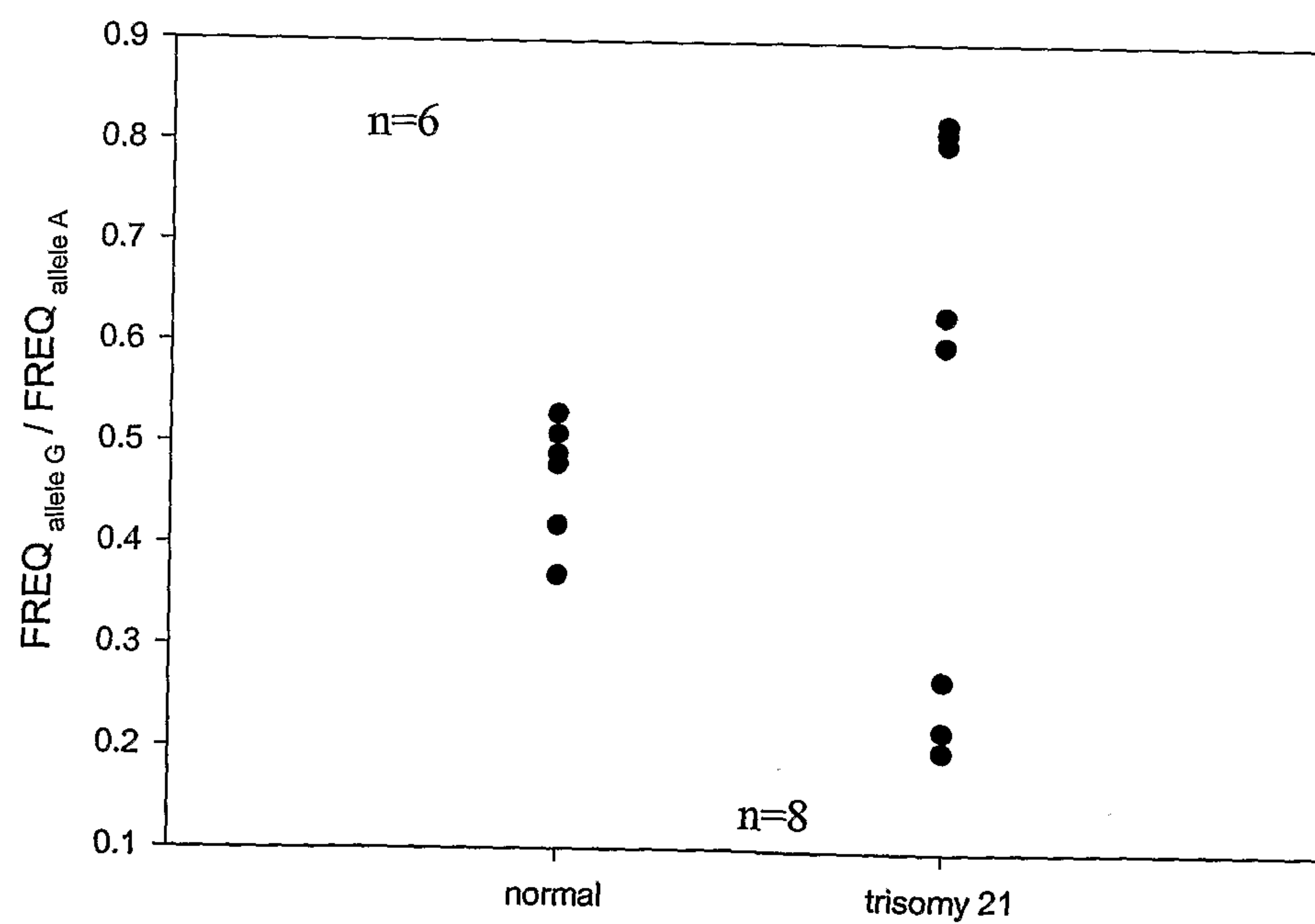


Figure 7

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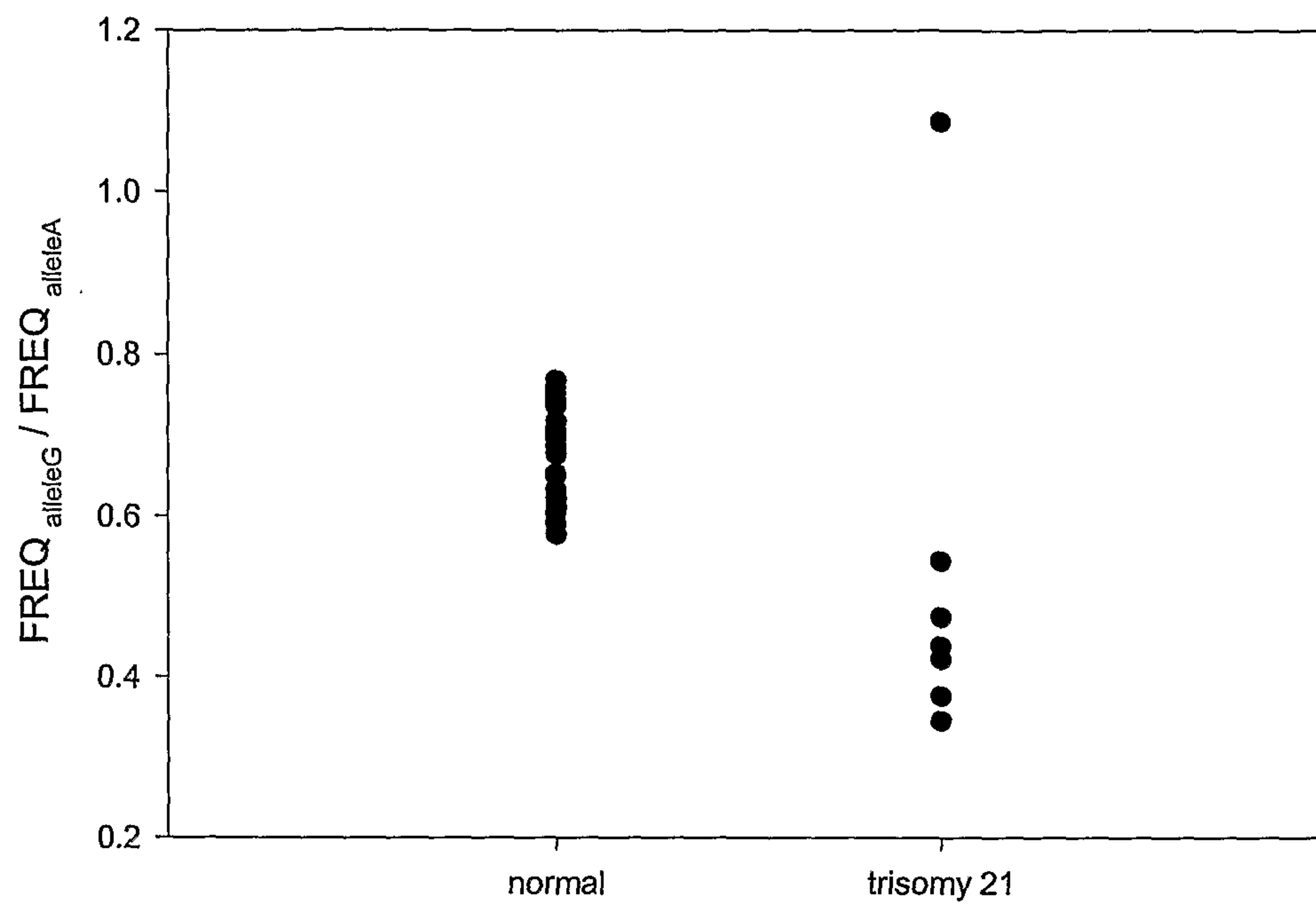


Figure 8

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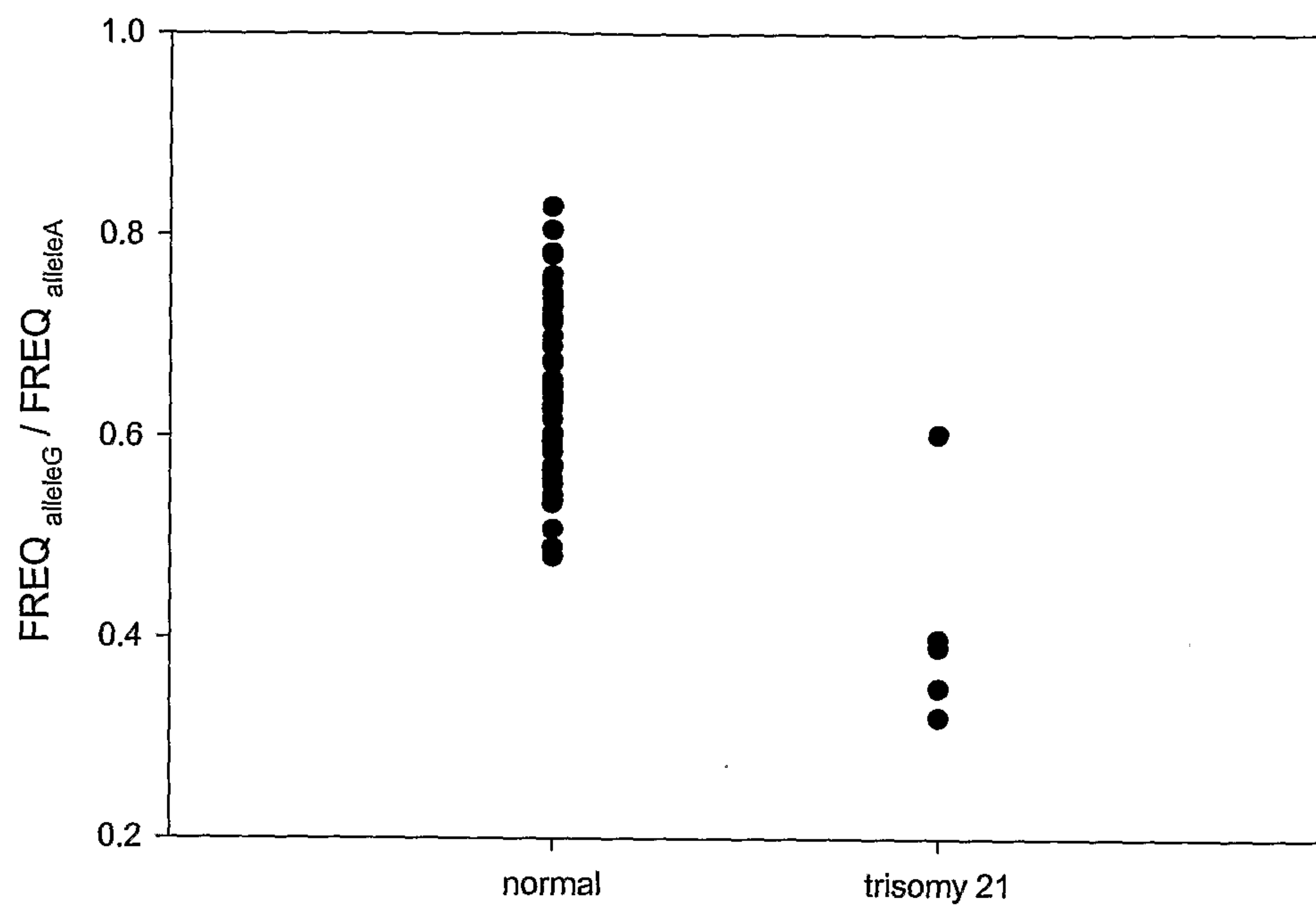


Figure 9

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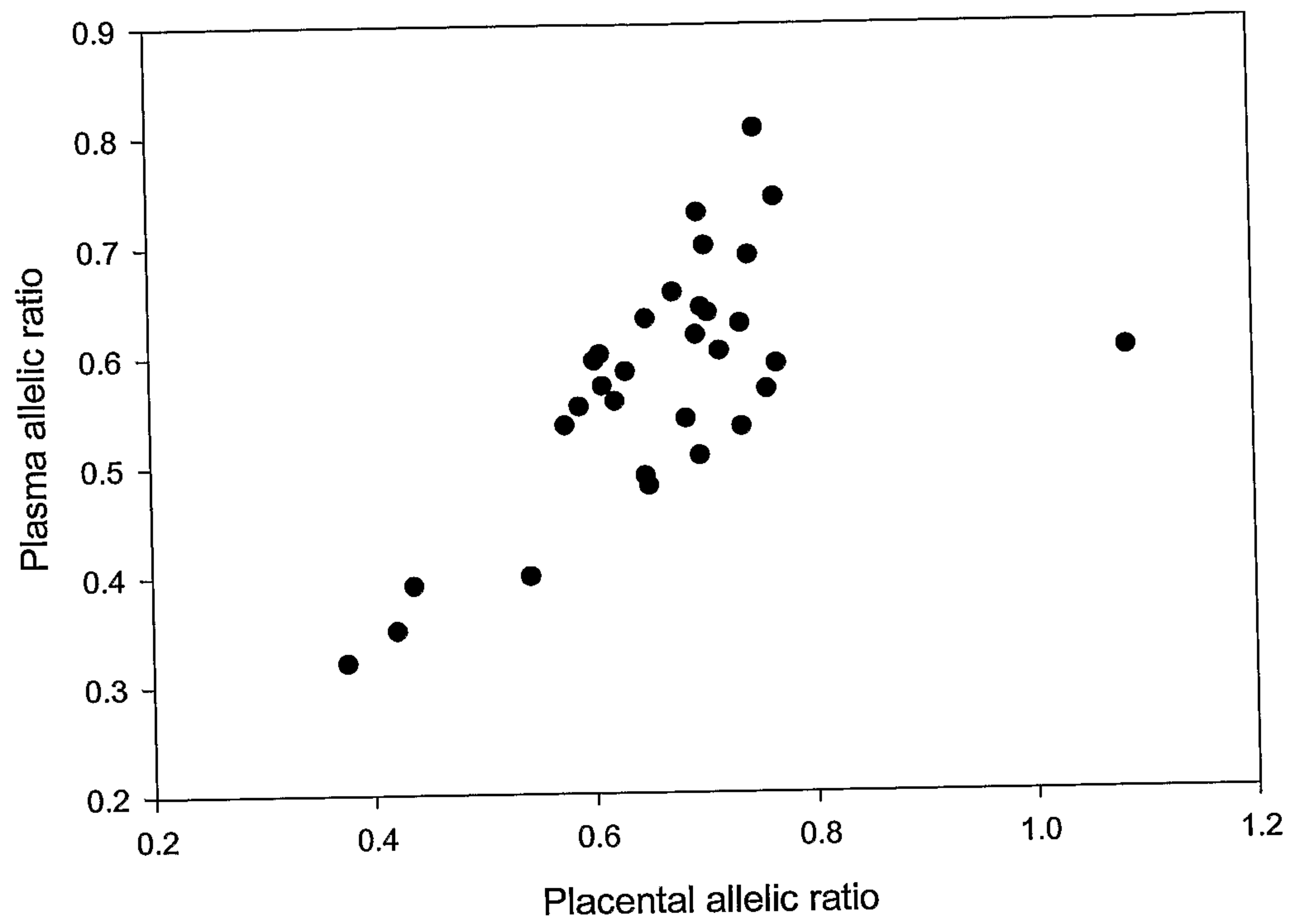


Figure 10

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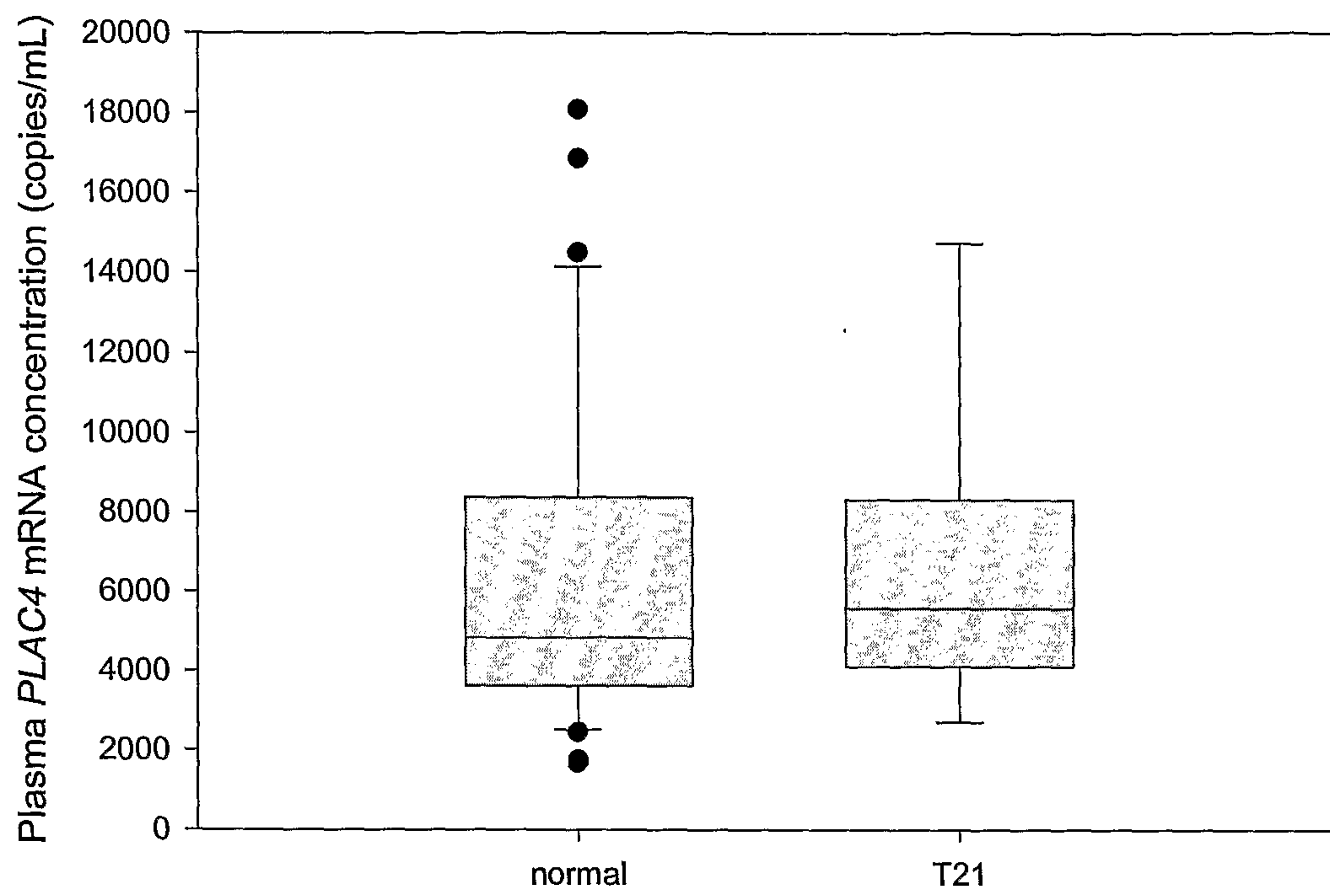


Figure 11

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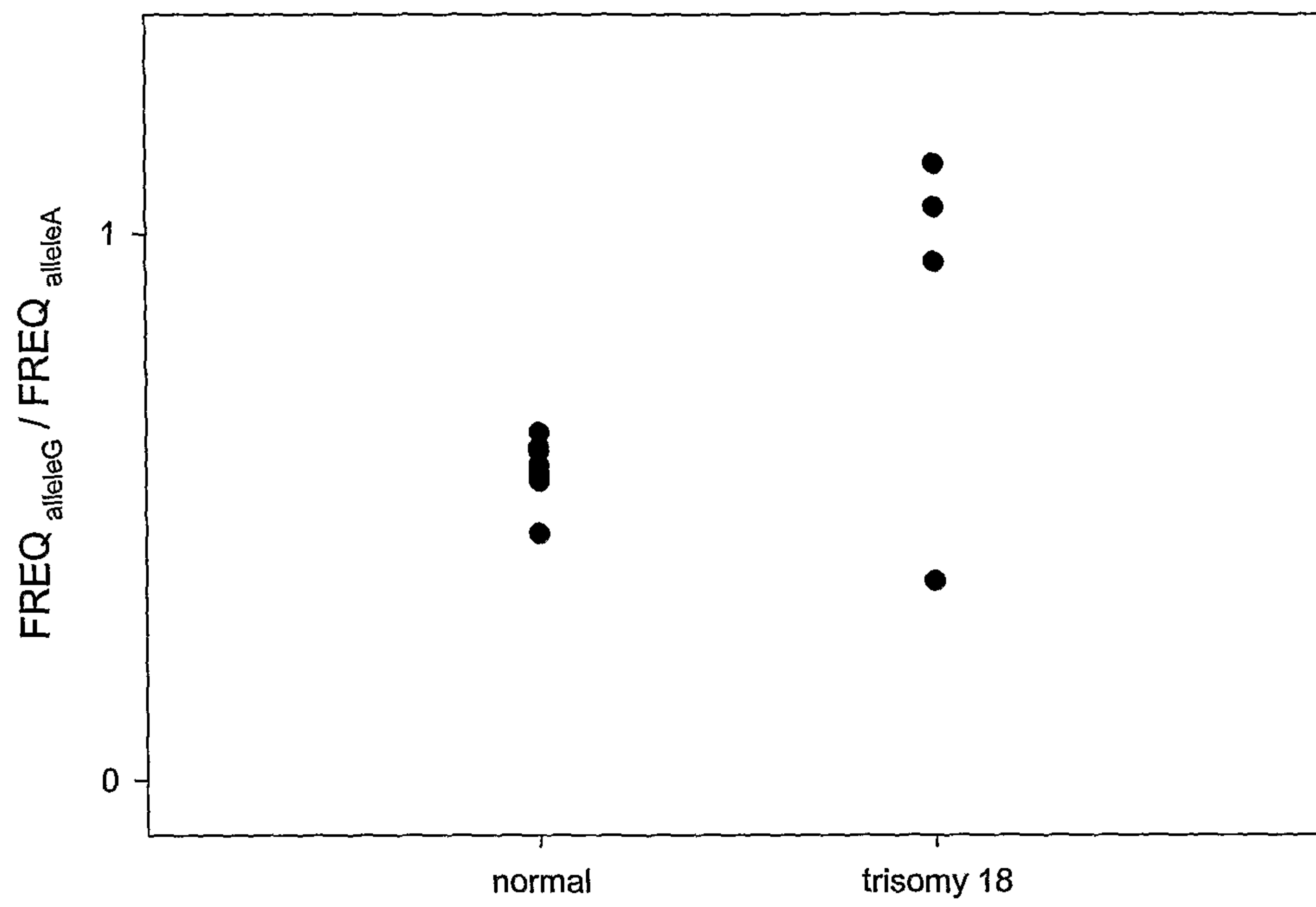


Figure 12

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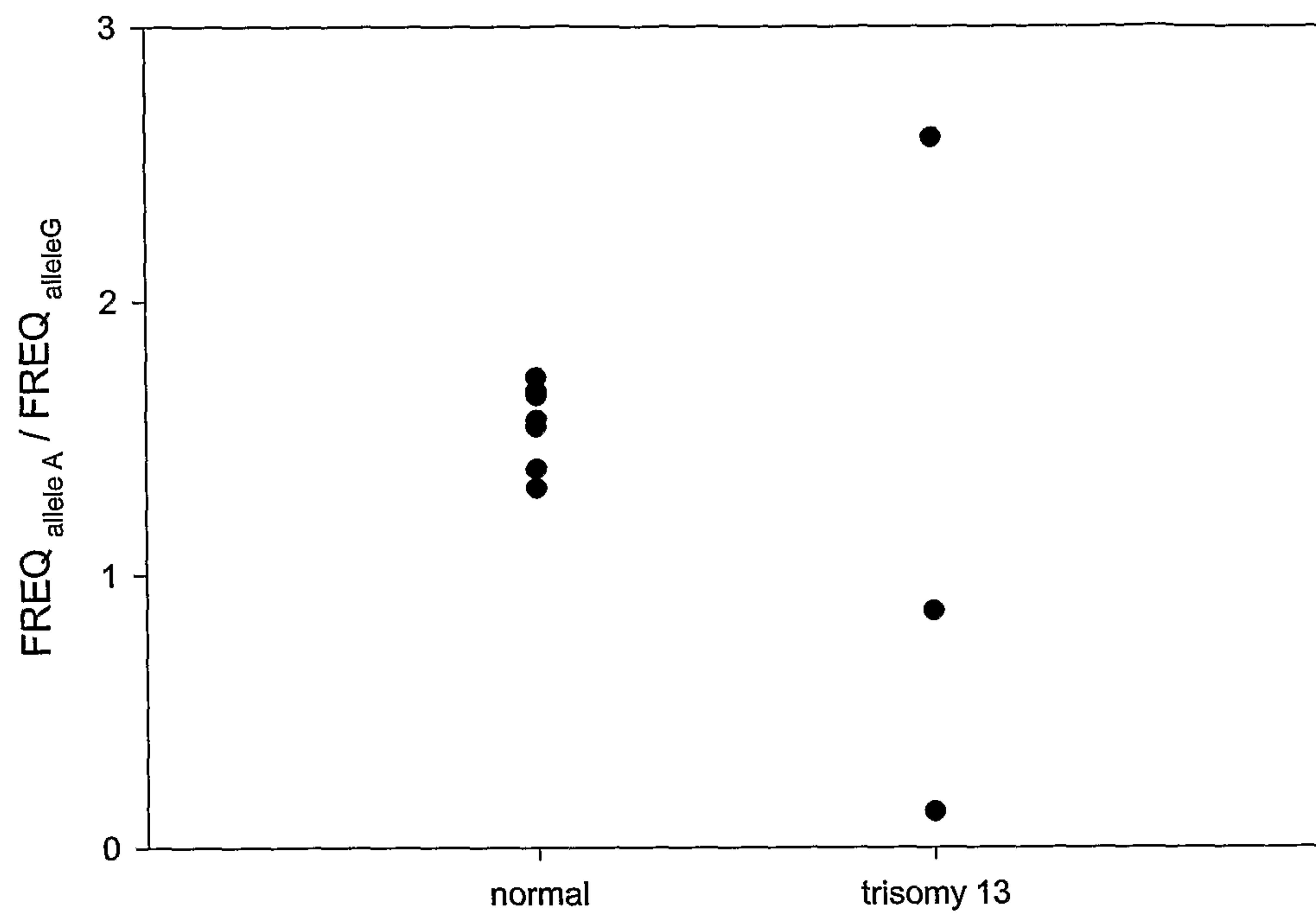


Figure 13