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with vitamin B_{12}, but both continue to have symptoms of peripheral neuropathy.

There have been reports of false normal results for vitamin B_{12} levels generated by automated analyzers when the serum of patients with megaloblastic anemia is evaluated. The results have been attributed to the possibility that high levels of intrinsic factor–blocking antibodies interfere with the assay.[1,2] Today, vitamin B_{12} assays are primarily performed on automated analyzers that apply a method based on the competitive binding of serum vitamin B_{12} with reagent intrinsic factor. Many of these platforms have also been found to be inaccurate when serum containing intrinsic factor–blocking antibodies is analyzed.[2] Disconcertingly, pernicious anemia is the most common cause of vitamin B_{12} deficiency, and up to 70% of patients with pernicious anemia have intrinsic factor–blocking antibodies.[3]

To investigate further, we precipitated serum immunoglobulins by adding 25% polyethylene glycol (PEG) by volume in a 1:1 dilution with serum. Using unmodified and PEG-treated samples of serum from the two patients and from three controls (patients without macrocytic anemia), we then ran tests for vitamin B_{12} levels (Fig. 1). In the PEG-treated samples from the two patients, vitamin B_{12} levels decreased to below the limit of detection; the PEG-treated samples from the controls showed a decrease compatible with the 1:1 dilution.

We have been performing vitamin B_{12} assays on the Siemens Dimension Vista system at our institution. A review of the package insert shows that the manufacturers are aware of this issue and recommend testing for intrinsic factor–blocking antibodies if test results are in conflict with the clinical diagnosis. We are in the midst of evaluating other platforms for this assay and have notified our clinicians of the issues described. However, we are concerned that there is insufficient awareness in the medical community of the possibility of spuriously high vitamin B_{12} levels; we urge pathologists to review their methods and clinicians to incorporate the information presented here into their diagnostic evaluations.

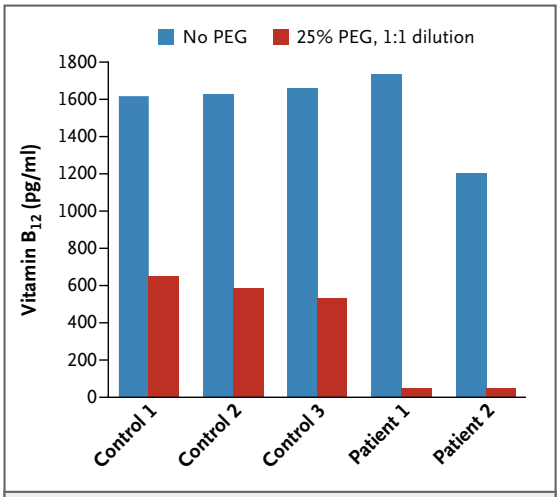

Figure 1. Spuriously Elevated Vitamin B_{12} Levels in Two Patients with Pernicious Anemia.

Vitamin B_{12} levels in serum samples to which polyethylene glycol (PEG) had been added and serum samples to which PEG had not been added are shown for two patients with pernicious anemia and three controls without anemia who had normocytic red cells. To convert the values for vitamin B_{12} to picomoles per liter, multiply by 0.7378.

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Disclosure forms provided by the authors are available with the full text of this letter at NEJM.org.

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A Noninvasive Test to Determine Paternity in Pregnancy

TO THE EDITOR: Five percent of women who are raped become pregnant, which results in an estimated 32,000 pregnancies annually in the United States.[1] In many circumstances, it is unclear whether the pregnancy resulted from the rape or from consensual intercourse. The only options available for prenatal paternity determination are invasive tests, such as the sampling of chorionic

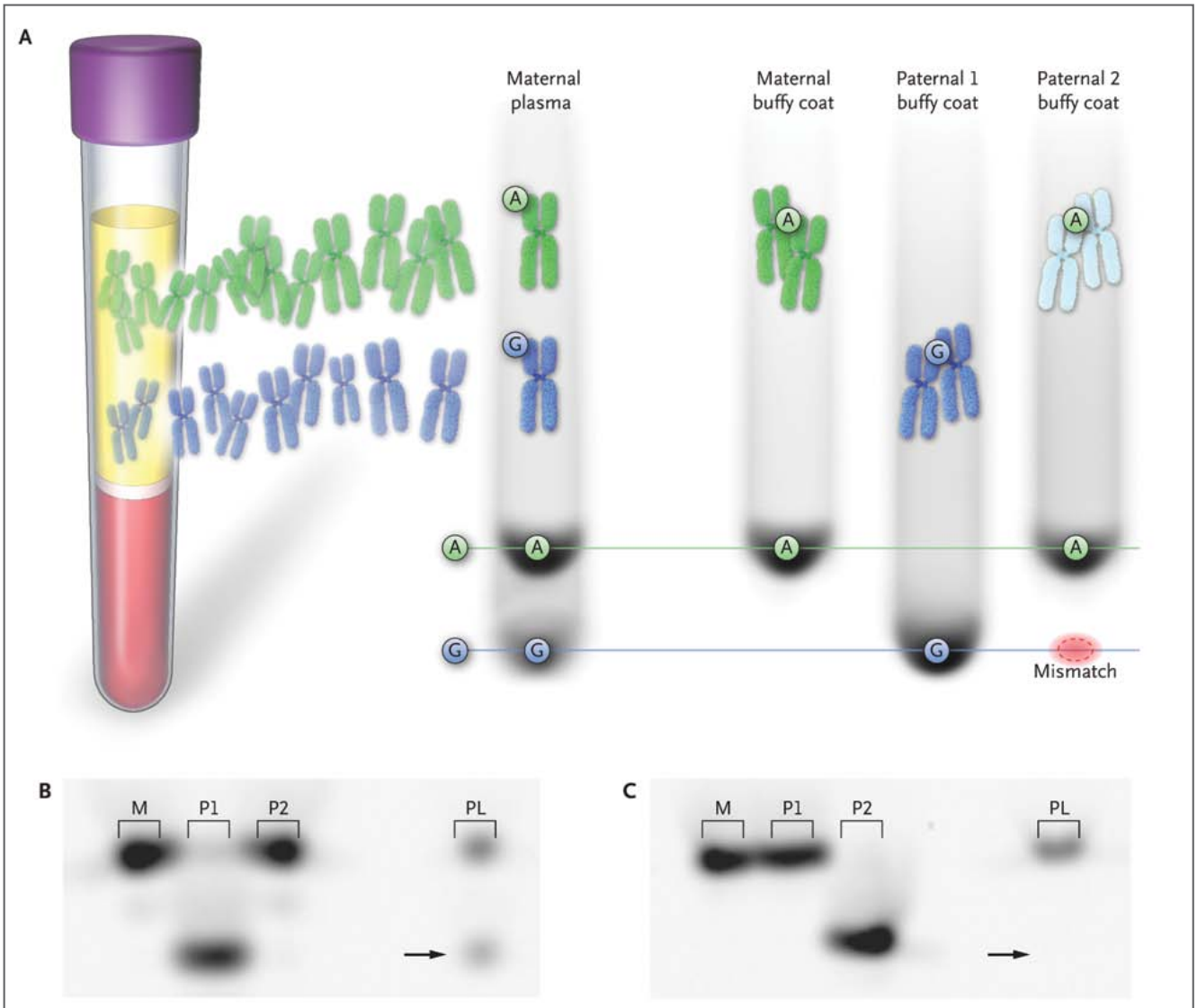

Figure 1. The Use of Informative Single-Nucleotide Polymorphisms (SNPs) to Determine Paternity.

An informative SNP is one in which the mother and one of the two potential fathers are homozygous for the same allele, whereas the other potential father is homozygous for the alternative allele. For example, the maternal (M) DNA and paternal 2 (P2) DNA in Panel A are homozygous (AA genotype) and the paternal 1 (P1) DNA is homozygous (GG genotype) at this informative SNP. In the maternal plasma (PL), the fetal DNA has the maternal allele (A allele) and paternal allele (G allele) at the informative SNP site. Paternal 2 does not have a G allele and therefore does not match the fetal DNA signal in maternal plasma. Paternal 2 can thus be excluded as a potential father. In Panels B and C, sequencing gel images of two informative SNPs show that the man with paternal 2 DNA cannot be the father and that paternal 1 is the biologic father of the fetus.

villi and amniocentesis, that carry a risk of miscarriage and are not performed before 10 to 15 weeks of gestation. Because 78.9% of terminations of unintended pregnancies are carried out before 10 weeks,[2] it seems likely that many rape victims terminate pregnancies before testing for paternity. A noninvasive prenatal paternity test based on cell-free fetal DNA present in maternal blood, performed at 8 weeks of gestation or later, could provide a safe option for determining paternity.

Previous studies of noninvasive prenatal paternity testing have shown that amplification of fetal alleles from maternal blood is suppressed by the presence of cell-free maternal DNA.[3] Furthermore, fetal DNA in maternal plasma is highly degraded. These limitations can be overcome by first adding a fixative to maternal blood samples

to stabilize cell membranes and prevent the release of maternal DNA into the plasma.[4] By using single-nucleotide polymorphisms to distinguish fetal DNA[5] from maternal DNA (Fig. 1), one can use short amplicons (shorter than 75 bp) to minimize allele dropout (absence of a fetal DNA signal when one should be present).

We collected blood samples from 30 women with pregnancies of 8 to 14 weeks of gestation. Each maternal blood sample was paired with blood from the biologic father and then randomly grouped with 1 of 29 samples from unrelated men. The 3 samples in each group were processed in a blinded manner. We determined paternity correctly for all 30 samples, by comparing the genetic profile of fetal DNA in maternal blood with those of the 2 "paternal" samples (1 genuine, 1 not) (Table 1 in the Supplementary Appendix, available with the full text of this letter at NEJM.org). The odds of identifying the correct father for all 30 samples are less than 1 out of 1 billion ($P=1.86\times10^{-9}$). Our approach shows that noninvasive prenatal paternity testing can be performed within the first trimester with the use of a maternal blood sample.

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Disclosure forms provided by the authors are available with the full text of this letter at NEJM.org.

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The annual EACME conference, entitled "Other Voices, Other Rooms: Bioethics, Then and Now," will be held in Bristol, England, Sept. 20–22.

Contact the School of Social and Community Medicine, Centre for Ethics in Medicine, Room G.04, Canynge Hall, Whatley Rd., Bristol BS8 2PS, United Kingdom; or call (44) 117 33 14521; or fax (44) 117 92 87326; or e-mail roz.hime@bristol.ac.uk; or see http://www.eacme2012.org.

Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Guo X, Bayliss P, Damewood M, et al. A noninvasive test to determine paternity in pregnancy. N Engl J Med 2012;366:1743-5.

Table of Contents

Table S1: Gestational age and data of informative SNPs for the 30 patient samples

Sample number	Estimated gestational age (weeks, days)	Number of SNPs mismatching the incorrect father (part one)	Number of non-specific bands in part one	Number of allele dropouts in part one	Number of additional SNPs matching the correct father (part two)	Total number of SNPs matching the correct father in part one and part two
1	13 3/7	6/6	0	0	13/13	16/16
2	11 5/7	4/6	2	0	15/16	18/19
3	9 4/7	6/6	0	0	11/12	14/15
4	12 1/7	6/6	0	0	13/13	16/16
5	8 5/7	6/6	0	0	13/14	16/17
6	13 3/7	6/6	0	0	14/14	17/17
7	11 5/7	5/6	0	1	12/12	14/15
8	11 1/7	6/6	0	0	15/16	18/19
9	10 5/7	5/6	1	0	15/15	18/18
10	10 4/7	6/6	0	0	12/12	15/15
11	11 1/7	6/6	0	0	17/17	20/20
12	8 1/7	6/6	0	0	14/16	17/19
13	11 3/7	6/6	0	0	13/13	16/16
14	14 2/7	6/6	0	0	15/15	18/18
15	10 2/7	5/6	0	1	11/11	13/14
16	8 6/7	6/6	0	0	12/12	15/15
17	11 0/7	6/6	0	0	12/12	15/15
18	13 0/7	4/6	2	0	13/13	16/16
19	8 6/7	4/6	2	0	15/15	18/18
20	8 0/7	5/6	1	0	10/11	13/14
21	10 4/7	6/6	0	0	19/20	22/23
22	9 1/7	4/6	2	0	15/16	18/19
23	8 6/7	6/6	0	0	14/15	17/18
24	10 3/7	6/6	0	0	13/13	16/16
25	9 4/7	6/6	0	0	16/16	19/19
26	9 0/7	5/6	1	0	15/16	18/19
27	9 5/7	6/6	0	0	11/11	14/14
28	12 0/7	6/6	0	0	17/17	20/20
29	8 0/7	5/6	1	0	14/14	17/17
30	12 2/7	5/6	0	1	15/15	17/18

Table S1 Legend:

Table 1 summarizes the data from the 30 study participants. Samples were collected from 2 clinical sites delivering out of Harrisburg Hospital in Pennsylvania from 10/2007 to 1/2010. Blood samples were collected from pregnant women and the stated biologic father. Following delivery buccal swabs from the neonates were collected to confirm paternity. The purpose of this non-invasive prenatal study to verify paternity was discussed during patient recruitment and in the informed consent. This was done to give women the opportunity not to participate, if there was a risk of non-paternity being discovered inadvertently as part of the study. Each maternal blood sample was paired with blood from the biologic father, and then randomly grouped with one of 29 samples from unrelated males, irrespective of ancestry which was provided by self report. A random number generator was used to assign the two potential fathers as p1 or p2 for each sample and sequentially processed in a blinded manner. To identify informative SNPs, maternal and paternal buffy coat DNA was genotyped at 384 SNPs. Amplification of SNPs was done by PCR utilizing an upstream primer that contained a recognition site for the restriction enzyme EcoRI and had a biotin tag at the 5′ end. The downstream primer contained a recognition site for a type IIS restriction enzyme. PCR products were bound to streptavidin-coated plates and then digested with a type IIS restriction enzyme such that the digest left a 5' over hang containing the SNP of interest and a 3' recessed end one base pair upstream of the SNP of interest. The SNP of interest was then filled in using fluorescently labeled dideoxy-NTPs to label the SNP. Following labeling, the PCR products were released by digest of EcoRI and loaded and run on a polyacrylamide gel and the gel was imaged using a Typhoon 9400 gel imaging system. This method, which was previously described in detail[1], has the primary advantage of both the PCR and restriction enzyme digest being highly specific along with not requiring hybridization at the SNP detection stage. The goal was to develop a cost effective test that could be run in any molecular biology laboratory.

Columns 3, 4, and 5 summarize the data from part one, in which the correct father was identified between two potential fathers using six informative SNPs as described here. Three of these SNPs were chosen so that the first potential father (p1) was homozygous for the alternative allele to that of the homozygous mother and the second potential father (p2) was homozygous for the same allele for which the mother was homozygous. This is illustrated in the diagram below.

	m	**p1**	**p2**	**f**
AA	⊗		⊗	⊗
aa		⊗		**?**

The remaining three SNPs had the homozygous allele matching the mother for p1 and the alternative allele to that of the mother for p2 as shown below.

	m	**p1**	**p2**	**f**
AA	⊗	⊗		⊗
aa			⊗	**?**

Exclusion of one of the two potential fathers was based on the decision rule of mismatch at 4 or more of 6 informative SNPs as compared to the observed fetal genotype in the maternal plasma. For 19 samples, 6 out of 6 informative SNPs mismatched; for 7 samples, 5 out of 6 informative SNPs mismatched; for 4 samples, 4 out of 6 informative SNPs mismatched. The median maternal age of the participants was 30 years (range 24 to 41 years); the median gestational age was 10

weeks and 4 days (Table 1). All but one of the study participants was in the first trimester (Sample #14 was 14 weeks and 2 days). Column 4 and 5, summarize the number of non-specific bands (NS) and allele dropouts (AD) seen in each sample in part one. In 4 samples, there was 1 non-specific band. In 4 samples, there were 2 non-specific bands. The average non-specific band rate (π_{NS}) is 13·3% (12 SNPs out of a total of 90 SNPs).

Part two is designed to show how one can test for paternity if only one potential father is available for testing. In part two of the study, an additional 11 to 20 informative SNPs were utilized per sample. The number of informative SNPs for a given sample varies depending on the specific genotype of the mother and potential father. Here informative SNPs are such that the father being tested is homozygous for the alternative allele to that of the mother, which is shown in the diagram below.

	m	**p1**	**f**
AA	⊗		⊗
aa		⊗	**?**

Column 6 shows the number of additional informative SNPs that matched the correct father. In 20 of 30 samples, all additional informative SNPs tested matched (i.e. no allele dropout); in 9 of 30 samples, all but 1 additional informative SNPs tested matched (i.e. 1 allele dropout); in 1 of 30 samples, all but 2 additional informative SNPs tested matched (i.e. 2 allele dropouts). Column 7 shows the total number of informative SNPs matching the correct father from part one and part two at which the mother and correct father were homozygous for alternate alleles. Among all 30 samples, a total of 426 additional informative opposite homozygous SNPs were utilized in part two. Among these 426 SNPs there were a total of 11 allele dropouts. Counting the three allele dropouts (Table 1, Column 5) from the 90 opposite homozygous SNPs tested in part one, the approximate allele drop out rate (π_{AD}) is 2·7% (14/516).

Table S2: Probability of paternity of the correct father versus the incorrect father

z_A	z_B	NS in exclusion	AD in exclusion	L (z_A, z_B)	W(z_A, z_B)
3	3	0	0	12660973.924	0.99999992
2	3	0	1	54322.291	0.99998159
3	2	1	0	54322.291	0.99998159
3	1	2	0	233.071	0.9957278

Table S2 Legend:

In part one, six informative SNPs were used to blindly identify 30 of 30 fathers between two potential fathers (p1 and p2). The table displays the probability of paternity, which is a likelihood ratio, for the correct father versus the incorrect father for a given number of non-specific bands and allele dropouts as observed in part one. The decision rule to exclude one of the two potential fathers is based on a mismatch at 4 or more of 6 informative SNPs as compared to the observed fetal genotype. The table shows that the probability of paternity is greater than 0.99 in all cases. For example, 19 samples had 6/6 SNPs mismatch the incorrect father with 0 NS and 0 AD and, thus, have a probability of paternity for the correct father of 0.99999992. Similarly, 7 samples had 5/6 SNPs mismatch the incorrect father with either 1 NS or 1 AD and, thus, have a probability of paternity for the correct father of 0.99998159. Finally, 4 samples had 4/6 SNPs mismatch the incorrect father with 2 NS and, thus, had a probability of paternity for the correct father of 0.9957278. The likelihood ratio of the observed data, L, and the probability of paternity, W, is calculated as follows. Under the a-priori equal likelihood assumption $W = \frac{L}{L+1}$. The likelihood ratio is defined as $L = \frac{\mathrm{P}(y|p1 \text{ is the father})}{\mathrm{P}(y|p2 \text{ is the father})}$, where $y = (zA; zB)$ is the observed data. When P1 is the father, $ZA \sim (Bin(3, (1 - \pi_{ad})))$ and $ZB \sim Bin(3, (1 - \pi_{ns}))$. But when P2 is the father $ZA \sim Bin(3, \pi_{ns})$ and $ZB \sim Bin(3, \pi_{ad})$.

So the likelihood ratio is $L(\pi_{ad}, \pi_{ns}; z_A, z_B) = \frac{(1-\pi_{ad})^{z_A}\pi_{ad}^{3-z_A} \times (1-\pi_{ns})^{z_B}\pi_{ns}^{3-z_B}}{\pi_{ns}^{z_A}(1-\pi_{ns})^{3-z_A} \times \pi_{ad}^{z_B}(1-\pi_{ad})^{3-z_B}}$.

Table S3: Probability of paternity for a tested father versus the untested world

Number SNPs	No exclusions	One exclusion	Two exclusions	Three exclusions	Four exclusions	Five exclusions	Six exclusions
3	0.8448	0.1581	0.0064	0.0002			
4	0.9054	0.2483	0.0113	0.0004	0.0000		
5	0.9439	0.3675	0.0197	0.0007	0.0000	0.0000	
6	0.9673	0.5055	0.0341	0.0012	0.0000	0.0000	0.0000
7	0.9812	0.6426	0.0584	0.0021	0.0001	0.0000	0.0000
8	0.9892	0.7598	0.0984	0.0038	0.0001	0.0000	0.0000
9	0.9938	0.8476	0.1611	0.0066	0.0002	0.0000	0.0000
10	0.9965	0.9073	0.2525	0.0115	0.0004	0.0000	0.0000
11	0.9980	0.9451	0.3726	0.0201	0.0007	0.0000	0.0000
12	0.9989	0.9680	0.5110	0.0348	0.0012	0.0000	0.0000
13	0.9994	0.9816	0.6476	0.0596	0.0022	0.0001	0.0000
14	0.9996	0.9894	0.7637	0.1004	0.0038	0.0001	0.0000
15	0.9998	0.9940	0.8504	0.1641	0.0067	0.0002	0.0000
16	0.9999	0.9966	0.9091	0.2566	0.0118	0.0004	0.0000
17	0.9999	0.9980	0.9462	0.3778	0.0205	0.0007	0.0000
18	1.0000	0.9989	0.9687	0.5164	0.0356	0.0013	0.0000
19	1.0000	0.9994	0.9820	0.6526	0.0609	0.0022	0.0001
20	1.0000	0.9996	0.9897	0.7677	0.1024	0.0039	0.0001
21	1.0000	0.9998	0.9941	0.8532	0.1671	0.0069	0.0002
22	1.0000	0.9999	0.9966	0.9109	0.2608	0.0120	0.0004
23	1.0000	0.9999	0.9981	0.9473	0.3829	0.0210	0.0007
24	1.0000	1.0000	0.9989	0.9694	0.5219	0.0363	0.0013
25	1.0000	1.0000	0.9994	0.9823	0.6575	0.0622	0.0023

Table S3 Legend:

Part two demonstrates how to generalize the method to test for paternity in instances where only one potential father is available for testing. The probability of paternity in this scenario can be calculated using this table and the formula described below. The table displays the probability of paternity applied to the likelihood ratio, over *n* (the number of SNPs) ranging from 3 to 25, and *zA* = *n*, *zA* = *n*–1 … *zA* = *n*–6, capturing no exclusions, one exclusion … six exclusions, respectively. For example, in part two, to reach a probability of paternity greater than 0.99 there need to be 9 SNPs that match the correct father with no exclusions; with one exclusion there need to be 14/15 SNPs that match the correct father; with two exclusions there need to be 19/21 SNPs that match the correct father. Under the usual independence and constant probability assumptions, given that p1 is the true father, then $ZA \sim Bin(n, (1 - \pi_{ad}))$. If this is not the true father, then the genotype possibilities for the true father are (assuming that genotypes are represented with probabilities, 0.25, 0.5 and 0.25 in the population respectively),

Prob.	**m**	**p1**	**p2**			**f**
			0.25	**0.5**	**0.25**	
AA	⊗		***AA***	***A***		⊗
aa		⊗		***a***	***aa***	**?**

There is a 50% chance that the unknown p2 contributes the "A" allele to the fetal DNA, and 50% that he contributes the "a" allele. For a single SNP, under the unknown father hypothesis, the probability that p1 is not excluded (matches) is *P*(p1 matches|p2 is the father) = 0.5**P*(p1 matches|p2 contributes "A")+0.5**P*(p1 matches|p2 contributes "a"). *P*(p1 matches|p2 is the father) = $0.5*\pi_{ns} + 0.5*(1 - \pi_{ad}) = 0.5*(1 + \pi_{ns} - \pi_{ad})$,which we define as π_u where "u" indicates denotes **u**nknown father. So if p1 is not the father, *ZA*, the number of matches to p1 follows the distribution $ZA \sim Bin(n, \pi u)$ and the likelihood ratio is $L = \frac{(1-\pi_{ad})^{z_A}\pi_{ad}^{n-z_A}}{\pi_u^{z_A}(1-\pi_u)^{n-z_A}}$.

Reference:

1. Dhallan R, Guo X, Emche S, et al. A non-invasive test for prenatal diagnosis based on fetal DNA present in maternal blood: a preliminary study. *Lancet* 2007; 369: 474–81.