B J O 0 9 6 0 | Journal Name | Manuscript No.
B | Dispatch: 11.4.06 | Journal: BJO | CE: Vidhya
Author Received: | No. of pages: 10 | ME: Varma

DOI: 10.1111/j.1471-0528.2006.00960.x
www.blackwellpublishing.com/bjog

Review article

HIV and reproductive care—a review of current practice

C Gilling-Smith,[a] JDM Nicopoullos,[a] AE Semprini,[b] LCG Frodsham[a]

[a] Assisted Conception Unit, Chelsea & Westminster Hospital, London, UK [b] Ospedale Luigi Sacco, Milan, Italy
Correspondence: Dr C Gilling-Smith, Assisted Conception Unit, Chelsea & Westminster Hospital, London SW10 9NH, UK.
Email cgs@chelwest.nhs.uk

Accepted 21 March 2006.

In developed countries, antiretroviral treatment has increased life quality and expectancy of HIV-infected individuals and led to a drop in mother-to-child transmission (MCT) risk to below 1%. Fertility has been shown to be reduced in both men and women with HIV. As a result of these factors, the demand for reproductive care in this population is rising. In discordant couples where the man is positive, sperm washing significantly reduces viral transmission risk to the uninfected female partner over unprotected intercourse. Positive women do not necessarily need specialised fertility treatment but should be monitored closely during pregnancy to minimise MCT risk.

Keywords Assisted reproduction, HIV, sperm washing, subfertility.

Please cite this paper as: Gilling-Smith C, Nicopoullos J, Semprini A, Frodsham L. HIV and reproductive care—a review of current practice. BJOG 2006; 113:1–10.

Introduction

HIV infection is a global epidemic that now affects more than 39 million people worldwide.[1] For the majority who live in the third world, the disease continues to record high fatalities through the inevitable progression of HIV to AIDS. In the developing world, reproduction is discouraged, although strong cultural pressures lead many to ignore government and medical advice on this matter, which adds further fuel to the epidemic by increasing sexual and vertical transmission rates. HIV infection further affects population decline by causing a relative subfertility, which has been widely reported in retrospective studies, but is of, as yet, unknown aetiology.[2]

By contrast, in the developed world, the introduction and development of highly active antiretroviral therapy (HAART) during the past decade has transformed the lives of those infected with HIV and led to its redefinition as a chronic, as opposed to fatal, disease. With continued improvements in HAART, projected life expectancy should approach that of negative controls. These changes mean that it is no longer justifiable to deny fertility treatment for HIV-positive adults, the majority of whom are of reproductive age.[3,4] In addition to improvements in life quality and expectancy, the use of selected antiretrovirals during pregnancy and at the time of delivery, elective caesarean section and the avoidance of breastfeeding are measures that have collectively led to a fall in vertical transmission risk from more than 30% to less than 2%.[5–10] As a result, increasing numbers of HIV-positive men and women are seeking advice on how to conceive with minimum risk of infecting their HIV-negative partner and prospective child or, in the case of concordant couples, of transmitting variant (drug resistant) viral strains to their partner or offspring.

It is difficult to gauge precisely the demand for reproductive assistance from HIV-positive patients. A recent UK audit of demand for assisted reproduction techniques in HIV-infected patients found that 16% of men and 4% of women attending HIV specialist clinics had enquired about fertility treatment. Following the Human Fertilisation and Embryology Authority (HFEA) recommendation of compulsory HIV, hepatitis B virus and hepatitis C virus screening prior to offering assisted reproduction techniques, 30% of fertility centres stated that they planned to start treating HIV-positive men and 26% stated that they planned to treat HIV-positive women.[11] Additional demand may be expected from assisted conception patients identified as HIV positive through screening. Hart *et al.*[12] in their audit of 2001 estimated this to be 1/1000 patients.

In this study, we review current practice and examine the three principal aspects of reproductive care pertinent to positive patients; reducing horizontal and vertical transmission of HIV to uninfected partner and prospective child, dealing with

any fertility issues and ensuring the safety of healthcare workers and other patients in the fertility centre.

Management of the positive men

Risks of unprotected intercourse

Reproductive assistance to HIV serodiscordant couples where the male partner is HIV positive and the female partner HIV negative can make a significant impact on the prevention of viral transmission. In such couples, it has been estimated that the HIV-negative female partner has a 0.1–0.5% risk of acquiring HIV per act of unprotected intercourse,[13,14] provided the couple are in a stable monogamous relationship, not abusing intravenous drugs or participating in any other form of high-risk activity. Viral load in semen correlates poorly with that in serum,[15,16] and men with undetectable plasma viral levels, such as those on HAART, can still transmit HIV in semen.[17] In an attempt to quantify the risk of timed unprotected intercourse in discordant couples trying to conceive, Mandelbrot *et al.*[18] studied 92 HIV-negative women with HIV-positive partners using this method alone. Four women seroconverted (4%), and although they were in relationships where the use of condoms outside the fertile window was inconsistent, the study points to the risks of unprotected intercourse timed to the fertile period. It has been argued that in men with an undetectable viral load, the risk of HIV transmission when unprotected intercourse is limited to the fertile window is very low, particularly if the couple have screened negative for genital infections. Attempting conception in this way is common practice in couples unable to access or finance risk-reduction options such as sperm washing. In a retrospective study of 77 discordant couples attempting to conceive in Spain, in whom the HIV-positive partner had undetectable HIV through use of HAART for at least 6 months, no seroconversions was noted.[19] The study did not analyse seroconversions in couples who failed to conceive, and overall, the numbers are too small to draw any valid conclusions on the safety of this approach. It is not possible to improve on safety by testing for HIV in semen as the detection of HIV RNA and DNA in ejaculated semen is unreliable.[20] Currently, HIV discordant couples where the male is infected and desires to eliminate or significantly reduce HIV transmission risk to their uninfected partner are limited to the following options:

1. Insemination using donor sperm: This effectively removes the risk of viral transmission as sperm donors are screened for HIV and other blood-borne viruses. However, it also removes the option of genetic parenting from the infected man.
2. Sperm washing: The female partner is inseminated with the infected partner's sperm, centrifuged first to separate spermatozoa from seminal fluid and associated nonsperm cells (NSC).
3. Adoption: While it is an acceptable route for those wishing to remove the risk of infecting their partners, current adoption practice regards HIV in one or both partners as a significant undesirable factor when assessing the suitability of parents requesting to adopt.

Sperm washing

Sperm washing rests on the observation, well supported in the literature, that HIV is present as free virus in the seminal plasma and as cell-associated virus in the leucocytes or NSC but does not appear to be able to attach to, or infect, the sperm.[21–26]

In a study conducted prior to establishing the sperm washing program at Chelsea and Westminster Hospital, levels of viral RNA, proviral DNA and expression of HIV receptors (CD4, CCR5 and CXCR4) were measured in semen centrifuged to separate into its different components. HIV RNA and proviral DNA were only found in seminal plasma and NSC, but spermatozoa themselves did not express significant levels of CD4, CCR5 and CXCR4, indicating that they are unlikely to be a major target for HIV infection.[25]

The process of sperm washing involves centrifuging the ejaculated semen in a 40–80% colloidal silica density gradient to separate progressively motile, HIV-free sperm from the infected NSC and seminal plasma that remain in the supernatant. The sperm pellet at the bottom is resuspended in fresh medium and centrifuged twice before preparation of a final swim-up. As a quality control for the procedure and to protect the service from medico-legal action, an aliquot of washed sperm (approximately 100 μl) should be tested for detectable HIV RNA prior to the sample being used for treatment.[27,28] A nucleic acid based sequence amplification (NASBA; Biomerieux, Basingstoke, Hampshire, UK)[29] or a similar commercial assay can be used.[30] The risk of the sample having detectable HIV is 5–6%.[27,31,32] The explanation for this is that in a small proportion of cases, centrifugation fails to remove all the seminal plasma and leucocytes. However, a limit is imposed on the number of washes as sperm damage during repeated centrifuging leads to loss of the sperm quality and quantity needed to achieve fertilisation. A modified 'double tube gradient' has been described to overcome the problem of failed washes and to improve the total sperm count in the final preparation.[33] A double tube is used, with the density gradient formed within the inner tube. As compared with the conventional sperm washing method described above, the double tube method was found to produce higher sperm yields in a comparative study using HIV-1 spiked semen samples. Nevertheless, the authors still recommend HIV testing of the washed sample.

This risk-reduction treatment, pioneered by Semprini *et al.*[34] in Milan, has a good safety record, with no reported cases of seroconversion in either female partner or child born in more than 3000 cycles of sperm washing combined

with intrauterine insemination (IUI), *in vitro* fertilisation (IVF) or intracytoplasmic sperm injection (ICSI) published to date[29,31,32,34–55] (Table 1).

Clinical work-up before sperm washing

Our recommendations are that all patients should receive careful preconceptual counselling, both together and individually before embarking on treatment.[27–29] Counselling should cover the nature and risks of sperm washing, the impact of possible treatment failure, coping with a child when one parent is HIV positive and the possibility of having to cope as a single parent. It is mandatory that both partners understand sperm washing to be a *risk-reduction* method and not a *risk-free* method as technically, the virus could still be present in the washed sample at a titre below the detection limit of the HIV assay (e.g. 25 viral copies/10^6 sperms in the case of the NASBA assay). Although there have been no reports of seroconversion in the female partner when semen is correctly processed,[27,28] the possibility of viral infection of the woman and subsequent child still exists, and the alternative risk-free option of donor insemination should be discussed. We advise centres to ensure that a consent form to the effect that sperm washing is a risk-reduction technique with no absolute guarantee of protecting the female partner from HIV is signed by both partners before treatment. This is an essential document that protects the service from medico-legal action should transmission occur.[27]

Apart from counselling, which is not universally offered, the majority of European centres follow very similar protocols for work-up prior to treatment. This involves a sexual health screen and fertility screen in both partners. The purposes of the sexual health screen are to first exclude the possibility of the female partner also being infected and the second is to treat any genital lesions or infections before treatment as these can increase the risk of viral transmission.[56] The aim of the fertility screen is to define the optimum mode of treatment. It is usual practice to perform a semen analysis, female endocrine profile and noninvasive test of tubal patency

55 **Table 1.** Published studies of sperm washing with assisted reproduction in HIV-positive men

		No. of couples treated	No. of inseminations/embryo transfers	Pregnancies (OP)	CPR (%)	OPR (%)	OPR/couple (%)
Semprini *et al.*[34]	IUI	29	59	17 (12)	28.8	20.3	41
Marina *et al.*[31,32]	IUI	63	101	31 (28)	30.7	27.7	44
	ICSI	1	1	1	—	—	—
Marina *et al.*[32]	IUI	—	303	88	29.0	—	—
Semprini *et al.*[50]	IUI	443	1461	216 (179)	14.8	12.3	40
Tur *et al.*[53,54]	IUI	64	155	32 (28)	20.6	18.1	35
Gilling-Smith[29]	IUI	19	40	8 (5)	20.0	12.5	26
Marina *et al.*[41]	IVF	—	29	12	41.4	—	—
Bujan *et al.*[35]	IUI	28	62	14 (13)	22.6	21.0	46
Guibert *et al.*[38]	ICSI	68	97	34 (25)	35.1	25.8	37
Loutradis *et al.*[40]	ICSI	2	2	2 (2)	—	—	—
Weigel *et al.*[55]	IUI	47	101	15 (13)	14.9	12.9	—
	IVF	5	10	6	60.0	—	—
	ICSI	14	21	9 (7)	—	—	
Bujan *et al.*[36]	IUI	39	93	18	19.4	—	—
Hanabusa *et al.*[39]	IVF	9	12	3 (3)	25	25	33
Pena *et al.*[45]	ICSI	2	2	2 (2)	—	—	—
Quintana *et al.*[47]	IUI	15	28	7 (7)	25.0	25.0	47
Sauer and Chang[48]	ICSI	34	55	25 (17)	45.5	30.9	40
Savasi *et al.*[49]	IUI	175	449	—	10.0	—	—
Semprini *et al.*[52]	ICSI	36	43	23 (20)	53.5	46.5	56
Marina *et al.*[42]	ICSI	156	219	92 (58)	42.0	30.6	59
Ohl *et al.*[44]	IUI	—	5	0	0	0	—
	ICSI	39	49	20 (14)	48.8	28.6	30.8
Pena *et al.*[46]	ICSI	61	96	43 (35)	44.8	36.5	50.8
Bujan *et al.*[37]	IUI	56	213	37 (30)	17.4	14.1	50
Nicopoullos *et al.*[43]	IUI	—	133	25	18.8	—	—

OP, ongoing pregnancy; OPR, ongoing pregnancy rate.

(e.g. hysterosalpingogram), unless there is a history of pelvic pain or infection where laparoscopy is the preferred method for assessing tubal patency.[27,57] Most couples embarking on sperm washing are voluntarily infertile and do not have significant fertility issues. For these couples, IUI is performed. At the Chelsea and Westminster Hospital, couples are offered IUI, provided the early follicular phase follicle-stimulating hormone (FSH) level is <12 units/ml, at least one tube is patent and the semen analysis shows normal parameters according to the criteria set by the World Health Organization (WHO).[58] If there is an evidence of spontaneous ovulation, insemination of washed sperm is carried out in a natural
4 cycle, monitored using ultrasound follicle tracking. In a similar fashion to donor insemination cycles, human chorionic gonadotrophin is administered to time insemination accurately. Ovulation induction, IVF or ICSI are combined with sperm washing when the fertility screen reveals anovulation, tubal blockage or suboptimal semen analysis, respectively.[27,29] A similar practice is adopted in the majority of European centres[28,57,59] in order to minimise the cost and risks associated with superovulation.

Sperm washing outcome in the UK

The Chelsea and Westminster Assisted Conception Unit has treated HIV-positive men, with sperm washing as part of risk-reduction program since 1999, and performed the largest series of inseminations in the UK. The results for IUI, IVF and ICSI using washed sperm in our series of 110 treated patients in April 2005 are shown in Table 2. All samples were demonstrated to have an undetectable viral load (<25 copies/ml), with a NASBA assay before treatment, and the IUI cycle cancellation rate due to a positive NASBA assay was 3.3% (7 of 213 cycles using fresh sperm samples). In all IVF/ICSI cycles, it was recommended that the couple had a postwash sample with an undetectable NASBA viral load cryopreserved as backup in case of a detectable NASBA test jeopardising the cycle on the day of egg collection. In this series, there have been no seroconversions in either female partner or child on rigorous follow up (HIV testing of female partner for up to 6 months after the last treatment with washed sperm). Satellite arrangements for investigations and follicle track scanning were arranged for geographical reasons (UK, Belgium, Ireland, Israel, Hong Kong, Canada) in several couples.[43] Although the majority of HIV-positive men had sperm parameters within the defined WHO normal range,[58] all parameters were significantly impaired when compared with HIV-negative controls and were found to correlate with CD4 cell count,[43] consistent with previous reports.[60] Despite these differences, pregnancy outcome achieved with IUI/sperm washing of 16.5% compares favourably with overall IUI live birth rates both nationally and in our unit. This has enabled us to confidently counsel couples that if the fertility screen indicates suitability for IUI, the effect of HIV does not seem to have a negative impact on pregnancy outcome.

56 **Table 2.** Pregnancy outcome in 110 HIV discordant couples treated with sperm washing (some couples with failed IUI cycles then had IVF or ICSI)

	IUI	IVF	ICSI
No. of treatment cycles started	213	43	50
No. of couples treated	74	30	31
Positive NASBA assay	7 (3.3%)	N/A	N/A
No. of inseminations/transfers	204	42	49
Age at time of treatment*	35 (23–49)	37 (25–42)	35.5 (22–42)
Biochemical pregnancy	35	17	6
% per insemination or transfer	17.2	40.5	12.3
Clinical pregnancy	32	16	6
% CPR/insemination or transfer	15.7	38.1	12.2
Miscarriages	9	1	0
Ectopic	0	1	0
OP	23	14	6
% OPR/insemination or transfer	11.3	33.3	12.2
Singleton delivery	14	7	4
Twin delivery	1	1	2
OP	8	6	0
% of couples pregnant	41.9 (31/74)	50.0 (15/30)	19.4 (6/31)
% of couples with OP	31.1 (23/74)	40.0 (12/30)	19.4 (6/31)

OP, ongoing pregnancy; OPR, ongoing pregnancy rate.
Hundred and ten couples treated overall (46.4% pregnant (51/110)/37.3% (41/110) OP).
*Values are represented as median (range).

Several determinants of success have previously been identified for IUI in HIV-negative men: the use of injectable gonadotrophins for superovulation in both unexplained infertility and mild male infertility; the use of clomifene citrate for superovulation in only unexplained infertility; maternal age; the number of preovulatory follicles; sperm parameters such as motility, morphology, postpreparation motility and, most consistently, total motile count inseminated. Based on our series of insemination cycles, we reported the first data to determine whether there are predictors of success in IUI/sperm washing in HIV-positive men.[43] A trend towards improved clinical pregnancy rate (CPR) with controlled ovarian stimulation was reported, but given the sample size, this did not achieve statistical significance. This supports our current practice of initially offering natural cycle of IUI for couples, unless anovulatory, before proceeding with ovulation induction after three to six failed cycles. We also reported a nonsignificant trend towards impaired outcome with increasing age. It may therefore also be reasonable to proceed with controlled ovarian stimulation and IUI immediately at advanced maternal ages. This option may be sensible in view of the cost involved per cycle and minimal funding currently available for the fertility treatment of HIV-infected couples. In our cohort of patients, the sperm parameters were not shown to have a significant impact on IUI outcome following sperm washing. Only markers of HIV infection significantly affected IUI outcome. CPR was significantly higher in cycles from men with low VL (<1000 copies/ml; 29 versus 11%, $P = 0.05$) and in those from men on antiretroviral therapy (27 versus 9%, $P = 0.02$). However, CD4 count had no impact on IUI outcome. It may therefore be sensible to advise HIV-positive men to commence antiretroviral medication to minimise viral load at the time of treatment. The exact mechanism by which HIV infection alters semen parameters remains unclear. Similarly, why CD4 count correlates with sperm parameters but only VL and the use of antiretrovirals predict IUI outcome also remains unclear and requires further investigation.

The outcome of ICSI treatment cycles shown in Table 2 includes the treatment of azoospermic HIV-positive men. In the first report of the use of surgical sperm retrieval, followed by sperm washing, we highlighted the difficulties of treating this group of men.[61] First, following the swim-up preparation of the semen sample, we recommended NASBA testing of 0.5×10^6 to 1×10^6 sperm cells. Considering the log loss of sperm during washing, an initial total sperm count of 5×10^6 is therefore usually recommended, which is often difficult to achieve, particularly where there is a nonobstructive cause of azoospermia. Second, from our experience, the high number of NSC found in the samples retrieved increases the risk of a positive postwash NASBA result. The subsequent risk of cancelled cycles can be minimised by having a cryopreserved sample as backup, but the quality of such samples is often questionable in view of requiring washing and testing prior to the freeze–thawing. Therefore, our current practice is to recommend a synchronous retrieval, with donor sperm backup to be used should either the retrieval fail to yield sperm or if the postwash NASBA test is positive.

Sperm washing in North America

Until recently, the American Society for Reproductive Medicine (ASRM) advised against the provision of assisted reproduction techniques, including sperm washing, to HIV-infected individuals. This followed a single report in 1990 of HIV transmission to a woman who underwent IUI using washed sperm from her HIV-positive husband.[62] The protocol recommended above was not adhered to, and the whole pellet postcentrifugation, including white cells, was inseminated.

The ASRM has now advocated a policy of nondiscrimination and equal access to fertility care,[63,64] and the first series in North America of sperm washing treatment in discordant HIV-positive men was published.[48] However, in this series, ICSI (without postwash testing) as opposed to insemination was used in all couples, despite the absence of male factor infertility. The authors argued that ICSI is the method of choice for assisted reproduction, regardless of sperm parameters, to minimise the exposure of each oocyte to one sperm rather than several million as is the case in IUI or IVF. We remain of the opinion that in order to safeguard patients from technical errors during washing, viral detection testing of semen samples prior to insemination is a necessary quality control step.[27,28] Aside from general concerns over cost, risks involved in ovarian stimulation cycles, multiple pregnancy risk and the long-term outcome of children born through ICSI, our worry over this approach is that HIV attached to the acrosome membrane could inadvertently be introduced into the oocyte during injection. In the light of the current data reported from European centres performing sperm washing, there appears to be no justification in offering sperm washing as part of an ICSI treatment in preference to IUI, unless a male fertility factor is identified.

Management of the positive woman

Demand for assisted conception

Retrospective data from the developing and developed world[2,65,66] and prospective data collected from our centre[11] have indicated relative subfertility in HIV-infected women. Our centre is the first to have demonstrated a significantly increased rate of tubal factor infertility in HIV-positive women;[11] 40 versus 14% in an unscreened subfertile population as demonstrated by Hull *et al.*[67] Sexual health screen was negative in all but two of our cohort of 57 HIV-positive women. These results are supported by evidence in the literature of increased severity of pelvic inflammatory disease (PID) in HIV-positive women[67] and high rates of PID from

genital tract commensals rather than demonstrated sexually transmitted infections.[68,69] These data alone would suggest that the demand for IVF in this group is set to rise. Menstrual cycle data in positive women does not suggest an increased incidence of oligomenorrhoea or amenorrhoea in positive women.[70,71] Likewise, a review of endocrine parameters in positive women indicates no significant differences in FSH and progesterone levels in positive women compared with that in negative controls.[72] However, two recent studies on positive women undergoing IVF in Spain suggest that HIV-positive women have lower success rates than HIV-negative women based on a reduced response to superovulation. These differences were not observed in the HIV-positive women undergoing ovum donation, pointing towards an effect of HIV on ovarian response and ovarian reserve rather than endometrial receptivity.[73] Although further prospective studies are needed to explore the effect of HIV on ovarian reserve and reproductive outcome, the data reviewed above points to increased tubal infertility and reduced ovarian reserve in positive women. It is therefore important that these women are referred for fertility evaluation at an early stage, particularly if there is a history of pelvic infection. The increased demand for reproductive care, particularly IVF, also needs to be addressed as current techniques used may need to be adapted to ensure minimal risk of viral transmission to offspring and other patients and staff in the fertility centre.

Reducing risk

In positive women, reducing vertical transmission risk is at present limited to measures taken during pregnancy and postnatally.[5–10] HIV-positive women planning assisted reproduction techniques should be offered an in-depth preconceptual counselling to discuss in detail the interventions required to reduce vertical transmission risk, their long-term health and the possible effects of antiretroviral medication on the fetus.[5,27] They are then advised on how to carry out self-insemination of their partner's sperm at the time of ovulation in order to minimise viral transmission risk through unprotected intercourse. As women are at increased risk of subfertility, we would recommend that fertility investigations are initiated if conception has not occurred within six cycles of self-insemination.[27] In order to ensure that vertical transmission risk is minimised, the physician for HIV and an obstetrician with experience in managing HIV-positive women should liaise closely and, if fertility treatment is required, the fertility centre should be part of this multidisciplinary team.

The impact of the invasive procedures used during assisted reproduction techniques on vertical transmission risk is unknown and the numbers treated so far are small. The invasive procedures required during IVF or ICSI have the potential of increasing viral transmission to the egg or the embryo. Gonadotrophin stimulation could increase viral load, oocyte retrieval through blood contamination could lead to HIV contamination of the oocyte and the embryos may become infected during passage of the transfer catheter. All these factors could enhance overall vertical transmission risk even when HAART is taken during pregnancy. However, it is likely that viral infection of the oocyte or the embryo at this early stage in development would reduce implantation rates and/or increase early pregnancy failure. HIV-positive women conceiving spontaneously are recognised to have an increased early miscarriage rate,[74] but miscarriage rates for positive women undergoing assisted conception have not yet been reported. Our centre has studied ten positive women undergoing IVF or ICSI and demonstrated that HIV was detectable in follicular fluid samples taken during routine vaginal egg collection in all patients ($n = 5$) with a detectable serum viral load and 60% (3/5) of those with an undetectable serum viral load. In addition, we demonstrated for the first time that there is a significant rise in serum viral load in superovulatory cycles versus unstimulated menstrual cycles. More reassuringly, only one lower genital tract sample of cervicovaginal secretions and endometrium from our patients ($n = 9$) had detectable virus.[75] This would indicate that if eggs were demonstrated free from virus prior to fertilisation, that embryo transfer may carry very little risk of HIV contamination. This would be in keeping with the recent IVF outcome data from Spain in HIV-positive women undergoing oocyte donation where pregnancy rate is similar to that in HIV-negative women.[73] However, numbers are small and further research in this field is required. Only one other article studying the effect of assisted conception on follicular fluid viral load exists and this showed an undetectable level of virus in all follicular fluid samples.[76] In this study, validation of the viral load assay did not appear to have been performed (as in our series), which could explain the discrepancy in the results.

These data emphasise the need for caution in offering assisted reproduction techniques to these women with close follow up to monitor pregnancy outcome and HIV status of the child. They also emphasise the need for centres electing to treat positive patients to be equipped with separate laboratory facilities to minimise HIV transmission risk to health workers, uninfected patients and their gametes and embryos.[77]

Clinical outcome

The number of assisted reproduction techniques centres treating positive women, discordant and concordant, is very small (a few centres in UK, France, Belgium and Spain) due to medico-legal and ethical restrictions in many countries. The Chelsea and Westminster Hospital has treated 22 positive women to date and results are shown in Table 3. Work-up, as with discordant couples planning sperm washing, includes preconceptual counselling, a sexual health screen and fertility screen on both partners. In concordant couples, sperm washing is advisable to prevent a mutated, drug-resistant viral

Table 3. Pregnancy outcome in 22 HIV-positive women treated at Chelsea and Westminster Hospital

	IUI	IVF	ICSI
No. of treatment cycles	23	22	8
No. of couples treated	8	10	6
Age at time of treatment*	34 (28–41)	35 (29–39)	36 (26–39)
Biochemical pregnancy	1	12	4
% per insemination or transfer	4.3	54.5	50.0
Clinical pregnancy	0	7	4
% CPR/insemination or transfer	—	31.8	50.0
Miscarriages	0	0	0
Ectopic	0	0	0
OP	0	7	4
% OPR/insemination or transfer	—	31.8%	50.0%
Singleton delivery	0	4	2
Twin delivery	0	0	0
OP	0	3	2
% of couples pregnant	12.5 (1/8)	70.0 (7/10)	50.0 (3/6)
% of couples with OP	(0/8)	60.0 (6/10)	50.0 (3/6)

OP, ongoing pregnancy; OPR, ongoing pregnancy rate.
Twenty-two couples treated overall (45.5% pregnant (10/22)/40.9% (9/22) OP).
*Values are represented as median (range).

strain being transmitted to the female partner during treatment. Case numbers in our series are too small to assess whether HIV infection has any impact on ovarian reserve and live birth rates.

Laboratory adaptations

The risk of cross-contamination to uninfected samples from negative patients or to health workers is increased when gametes and embryos from positive men and women are handled in the same laboratory. For this reason, we have proposed that samples from patients with known or suspected blood-borne viruses are handled separately both in time and space. The latter is best addressed by providing a separate laboratory with equipment (incubators, flow hoods, storage tanks, etc.) dedicated to handling infected samples.[77] With respect to cryopreservation, many European centres recommend that sperm and embryos from patients with HIV or other known viral infections such as hepatitis B or hepatitis C should be cryopreserved in heat-sealed straws in a separate tank.[78] In the UK, the HFEA does not advocate this method and has advised that samples from patients with known viral infections are stored in a separate tank for each infection or infection combination. There are arguments for moving all samples to vapour phase storage, noninfected and infected, to minimise transmission risk, although the long-term effects of these methods on embryo outcome is still being evaluated.[79] These proposals remain a matter of debate, and the European Directive to be published later this year may well provide formal guidance on the handling of known infected samples in the assisted conception setting.[80]

Ethical and medico-legal aspects of offering treatment

For many positive men and women, the doors to receiving risk-reduction reproductive assistance remain closed, as centres electing to treat this group of patients are few and predominantly located in Europe. These barriers to treatment are predominantly ethical and medico-legal, rather than practical, and both issues continue to be extensively debated.[3,4,63–65,81–87]

Few question offering treatment to men with stable disease where the sole purpose is to reduce transmission risk to the uninfected partner or child through sperm washing or donor insemination. More questionable is providing IVF or ICSI treatment to positive women or concordant couples with fertility issues or unstable disease. Vertical transmission risk, quoted as <1% with intervention, multiresistant disease or AIDS defining symptoms are regarded by some as unacceptable determinants in the decision to assist reproduction. Our view, and that shared by a number of European centres, is that positive patients should not be discriminated against and instead be provided with the opportunity to have children safely. Nevertheless, this is no 'Carte Blanche' for all and criteria such as viral load, CD4 count, presence of AIDS defining symptoms, stability of the relationship and associated high-risk behaviour should be assessed through appropriate preconceptual counselling and, if necessary, the local ethics

committee. In North America, the majority of assisted reproduction techniques centres have been opposed to treating infected patients in the past, but attitudes are changing, with a limited number now offering sperm washing to HIV-infected men,[48] although none so far has offered treatment to positive women.

The European network and database

In June 2003, a network linking all the major centres in Europe providing assisted reproduction techniques to HIV discordant couples through semen washing was founded in Madrid. The organisation is called CREATE—Centres for REproductive Assistance Techniques to HIV couples in Europe, which links centres in the UK, Italy, France, Belgium, Spain, Italy, Switzerland and Israel. The main objectives of this European database are to promote sperm washing and other assisted reproduction techniques to couples affected by HIV (and other sexually transmitted infections) and assist clinical centres wishing to establish or to have access to these services and provide guidelines based on combined experience. A further benefit is to pool data to establish the safety of methods used as the numbers of cases of men, and in particular women, being treated are still small. The combined database of CREATHE has analysed retrospectively 4989 sperm washing cycles carried out by member centres since 1989 and can confirm that more than 500 babies have been born with no reports of neonatal or seroconversion in uninfected partner.[28] However, serological follow up in some centres has been incomplete and the network plans in future to collect data prospectively with rigorous follow up of uninfected women and offspring. Nevertheless, these data are reassuring and should encourage other centres in geographically strategic locations across the world to set up similar services and improve access to a treatment, which, in both the short and long term, will reduce the spread of HIV in the heterosexual population. ■

References

1 UNAIDS. *Report on the Global AIDS Epidemic. Executive Summary.* 2004.

2 Glynn JR, Buve A, Carael M, Kahindo M, Macauley IB, Musonda RM, *et al*. Decreased fertility among HIV-1 infected women attending antenatal clinics in three African cities. *J Acquir Immune Defic Syndr* 2000;25:345–52.

3 Gilling-Smith C, Smith JR, Semprini A. Infertility and HIV: time to treat. *Br Med J* 2001;322:566–7.

4 Englert Y, Van Vooren JP, Place I, Liesnard C, Laruelle C, Delbaere A. ART in HIV-infected couples. Has the time come for a change in attitude? *Hum Reprod* 2001;16:1309–15.

5 BHIVA Guidelines Writing Committee. Guidelines for the management of HIV infection in pregnant women and the prevention of mother-to-child transmission of HIV. *HIV Med* 2005;6(Suppl 2):107–48.

6 Coll O, Fiore S, Floridia M, Giaquinto C, Grosch-Worner I, Guiliano M, *et al*. Pregnancy and HIV infection: a European consensus on management. *AIDS* 2002;16(Suppl 2):S1–18.

7 European Collaborative Study. Mother to child transmission of HIV infection in the era of highly active antiretroviral therapy. *Clin Infect Dis* 2005;40:458–65.

8 Sullivan JL. Prevention of mother-to-child transmission of HIV—what next? *J Acquir Immune Defic Syndr* 2003;34(Suppl 1):S67–72.

9 The International Perinatal HIV Group. Mode of delivery and vertical transmission of HIV-1: a meta-analysis from fifteen prospective cohort studies. *N Engl J Med* 1999;340:977–87.

10 Thorne C, Newell ML. Prevention of mother-to-child transmission of HIV infection. *Curr Opin Infect Dis* 2004;17:247–52.

11 Frodsham LCG, Boag F, Barton S, Gilling-Smith C. HIV positive fertility care for couples in the UK—supply and demand. *Fertil Steril* (in press).

12 Hart R, Khalaf Y, Lawson R, Bickerstaff H, Taylor A, Braude P. Screening for HIV, hepatitis B and C infection in a population seeking assisted reproduction in an inner London hospital. *BJOG* 2001;108:654–6.

13 De Vincenzi I. A longitudinal study of human immunodeficiency virus transmission by heterosexual partners. *N Engl J Med* 1994;331:341–6.

14 Gray RH, Wawer MJ, Brookmeyer R, Sewankambo NK, Serwadda D, Wabwire-Mangen F, *et al*. Probability of HIV-1 transmission per coital act in monogamous, heterosexual, HIV-1 serodiscordant couples in Rakai, Uganda. *Lancet* 2003;357:1149–53.

15 Luizzi G, Chirianni A, Clement M, Bagnarelli P, Valenza A, Cataldo PT, *et al*. Analysis of HIV-1 load in blood, semen and saliva: evidence for different viral compartments in a cross-sectional and longitudinal study. *AIDS* 1996;10:F51–6.

16 Coombs RW, Speck CE, Hughes JP, Lee W, Sampoleo R, Ross SO, *et al*. Association between culturable human immunodeficiency virus type 1 (HIV-1) in semen and HIV-1 RNA levels in semen and blood: evidence for compartmentalisation of HIV-1 between semen and blood. *J Infect Dis* 1998;177:320–30.

17 Zhang H, Domadula G, Beumont M, Livornese L Jr, Van Uitert B, Henning K, *et al*. Human immunodeficiency virus type 1 in the semen of men receiving highly active antiretroviral therapy. *N Engl J Med* 1998;339:1803–9.

18 Mandelbrot L, Heard I, Henrion-Geant R, Henrion R. Natural conception in HIV-negative women with HIV-infected partners. *Lancet* 1997;349:850–1.

19 Barreiro P, Soriano V, Nunez M, Gonzalez-Lanoz J. *Benefit of antiretroviral therapy for serodiscordant couples willing to be parents*. 7th International Congress on Drug Therapy in HIV Infection; 2004.

20 Dunne AL, Mitchell F, Allen KM, Baker HW, Garland S, Clarke GN, *et al*. Analysis of HIV-1 viral load in seminal plasma samples. *J Clin Virol* 2003;26:239–45.

21 Baccetti B, Benedetto A, Burrini AG, Collodel G, Elia C, Piomboni P, *et al*. HIV particles detected in spermatozoa of patients with AIDS. *J Submicrosc Cytol Pathol* 1991;23:339–45.

22 Bagasra O, Farzadegan H, Seshamma T, Oakes JW, Saah A, Pomerantz RJ. Detection of HIV-1 proviral DNA in sperm from HIV-1 infected men. *AIDS* 1994;8:1669–74.

23 Quayle AJ, Xu C, Mayer KH, Anderson DJ. T lymphocytes and macrophages, but not motile spermatozoa, are a significant source of human immunodeficiency virus in semen. *J Infect Dis* 1997;176:960–8.

24 Quayle AJ, Xu C, Tucker L, Anderson DJ. The case against an association between HIV-1 and sperm: molecular evidence. *J Reprod Immunol* 1998;41:127–36.

25 Kim LU, Johnson MR, Barton S, Nelson MR, Sontag G, Smith JR, *et al*. Evaluation of sperm-washing as a potential method of reducing HIV transmission in HIV-discordant couples wishing to have children. *AIDS* 1999;10:F51–6.

26 Vernazza PL, Gilliam BL, Dyer J, Fiscus SA, Eron JJ, Frank AC, *et al*. Quantification of HIV in semen: correlation with antiviral treatment and immune status. *AIDS* 1997;11:987–93. 22

27 Gilling-Smith C, Almeida P. HIV, hepatitis B & hepatitis C and infertility: reducing risk. Educational Bulletin sponsored by the Practice & Policy Committee of the BFS. *Hum Fertil* 2003;6:106–12.

28 Semprini AE, Fiore S. HIV and reproduction. *Curr Opin Obstet Gynecol* 2004;16:257–62.

29 Gilling-Smith C. Assisted reproduction in HIV discordant couples. *AIDS Read* 2000;10:581–7.

30 Burgisser P, Vernazza P, Flepp M, Boni J, Tomasik Z, Hummel U, *et al*. Swiss Cohort Study: performances of five different assays for the quantification of viral load in persons infected with various subtypes of HIV-1. *J Acquir Immune Defic Syndr* 2000;23:138–44. 23

31 Marina S, Marina F, Alcolea A, Exposito R, Huguet J, Nadal J, *et al*. Human immunodeficiency virus type I-serodiscordant couples can bear healthy children after undergoing intrauterine insemination. *Fertil Steril* 1998;70:35–9. 24

32 Marina S, Marina F, Alcolea R, Nadal J, Exposito R, Huguet J. Pregnancy following intracytoplasmic sperm injection from an HIV-1 seropositive man. *Hum Reprod* 1998;13:3247–9. 25

33 Politch JA, Su C, Tucker L, Anderson DJ. Separation of human immunodeficiency virus type 1 from motile sperm by the double tube gradient method versus other methods. *Fertil Steril* 2004;81: 440–7.

34 Semprini AE, Levi-Setti P, Bozzo M, Ravizza M, Taglioretti A, Sulpizio P, *et al*. Insemination of HIV-negative women with processed semen of HIV-positive partners. *Lancet* 1992;340:1317–19. 26

35 Bujan L, Daudin M, Righi L, Berges L, Thauvin L, Mieusset R. Effectiveness of "sperm-washing" to recover spermatozoa without HCV and HIV genomes detection in HIV infected men (Abstract). 4th International Symposium on AIDS and Reproduction. 2001. 27

36 Bujan L, Daudin M, Pasquier C. Reproductive options for HIV-discordant couples (Letter). *Perspect Sex Reprod Health* 2002;34:104.

37 Bujan L, Pasquier C, Labeyrie E, Lanusse-Crousse P, Morucci M, Dadin M. Insemination with isolated and virologically tested spermatozoa is a safe way for human immunodeficiency type 1 virus-serodiscordant couples with an infected male partner to have a child. *Fertil Steril* 2004; 82:857–62.

38 Guibert J, Merlet F, Le Du A, *et al*. ICSI for HIV-1 serodifferent couples: results of a preliminary study. *Hum Reprod* 2001;17(Suppl):56–7. 28

39 Hanabusa, *et al*. Clinical results of IVF using modified swim-up technique to remove HIV from semen (Abstract). *J Jpn Soc AIDS Res* 2002;4:S14–18. 29

40 Loutradis D, Drakakis P, Kallianidis K, Patsoula E, Bletsa R, Michalas S. Birth of two infants who were seronegative for human immunodeficiency virus type 1 (HIV-1) after intracytoplasmic injection of sperm from HIV-1 seropositive men. *Fertil Steril* 2001;75:210–2. 30

41 Marina F, Alcolea R, Exposito R, Martin P, Fosas N, Perez N, *et al*. Special semen-washing technique as a safe method for using assisted-reproduction techniques in HIV-1 seropositive men. *Hum Reprod* 2000; 15(Suppl):155–6. 31

42 Marina S, Semprini AE, Marina F, Vuccetich A, Alcolea R, Fosas N, *et al*. Results of 219 IVF-ICSI cycles in serodiscordant couples (seropositive men) to HIV-1. *Hum Reprod* 2003;18 S1:xviii152. 32

43 Nicopoullos JDM, Ramsay JWA, Almeida PA, Gilling-Smith C. The effect of HIV on sperm parameters and the outcome of IUI following sperm-washing. *Hum Reprod* 2004;19:2289–97.

44 Ohl J, Partisani M, Wittemer C, Schmitt MP, Cranz C, Stoll-Keller F, *et al*. Assisted reproduction techniques for HIV serodiscordant couples: 18 months of experience. *Hum Reprod* 2003;18:1244–9. 33

45 Pena JE, Klein J, Thornton M, Chang PL, Sauer MV. Successive pregnancies with delivery of two healthy infants in a couple who was discordant for human immunodeficiency virus infection. *Fertil Steril* 2002;78:421–3.

46 Pena JE, Thornton MH, Sauer MV. Assessing the clinical utility of in vitro fertilization with intracytoplasmic sperm injection in human immunodeficiency virus type 1 serodiscordant couples: report of 113 consecutive cycles. *Fertil Steril* 2003;80:356–62. 34

47 Quintana R, Tiveron M, Garcia F, Kopcow L, BisioliC, Young E. Intrauterine insemination (IUI), in vitro fertilisation (IVF) and intracytoplasmic sperm injection (ICSI) in human immunodeficiency virus (HIV) serodiscordant couples: results and perspectives. *Fertil Steril* 2002; 78:S116.

48 Sauer MV, Chang PL. Establishing a clinical program to assist HIV-1 seropositive men to have children using IVF-ICSI. *Am J Obstet Gynecol* 2002;186:627–33.

49 Savasi V, Persico T, Oneta M, Lanzani C, Crivelli M, Grandi MD. Intrauterine insemination in HIV-serodiscordant couple for male HIV infection (Abstract). *Int Conf AIDS* 2002;14. 35

50 Semprini AE, Fiore S, Oneta M, *et al*. Assisted reproduction in HIV-discordant couples. *Hum Reprod* 1998;13(Suppl):89. 36

51 Semprini AE, Vucetich A, Oneta M, *et al*. IVF-ET with processed sperm of HIV positive males in infertile HIV-discordant couples. *Fertil Steril* 1999;72:S40. 37

52 Semprini AE, Vucetich A, Oneta M, Rezek, D, Rubino P, Scarselli F, *et al*. Sperm-washing and ICSI in HIV discordant couples: >50% pregnancy rate (Abstract P-340). *Hum Reprod* 2002;17(Suppl):117–18.

53 Tur R, Veiga A, Busquets A, *et al*. Artificial insemination with processed sperm samples from serodiscordant couples for HIV-1. *Hum Reprod* 1999;14(Suppl):208. 38

54 Veiga A, Coll O, Tur R, Busquets A, Barri PN. Assisted reproductive technologies and HIV-1 serodiscordant couples. *Prenat Neonatal Med* 1999;4:356–61.

55 Weigel M, Gentili M, Beichert M, Friese K, Sonnenberg-Schwan U. Reproductive assistance to HIV-discordant couples—the German approach. *Eur J Med Res* 2001;6:259–62.

56 Fleming DT, Wasserheit JN. From epidemiological synergy to public health policy and practice: the contribution of other sexual transmitted diseases to sexual transmission of HIV infection. *Sex Transm Infect* 1999;75:3–17.

57 Weigel MM, Kremer H, Sonnenberg-Schwan, Golz J, Gurtler L, Doerr HW, *et al*. Diagnostics and treatment of HIV-discordant couples who wish to have children. *Eur J Med Res* 2001;6:317–21. 39

58 World Health Organization. *WHO Laboratory Manual for the Examination of Human Semen*. Cambridge, UK: 2000. 40

59 Semprini AE, Fiore S, Pardi G. Reproductive counselling for HIV discordant couples. *Lancet* 1997;349:1401–2.

60 Dulioust E, Le Du A, Costagliola D, Guibert J, Kunstmann JM, Heard I, *et al*. Semen alterations in HIV-1 infected men. *Hum Reprod* 2002; 17:2112–18. 41

61 Nicopoullos JDM, Frodsham LCG, Ramsay JWA, Almeida PA, Rozis G, Gilling-Smith C. Synchronous sperm retrieval and sperm washing in an ICSI cycle in an azoospermic HIV positive man: a case report. *Fertil Steril* 2004;81:831–4. 42

62 Centers for Disease Control. HIV-1 infection and artificial insemination with processed sperm. *MMWR Morb Mortal Wkly Rep* 1990;39: 249–56.

63 Ethics Committee of the American Society for Reproductive Medicine. Human immunodeficiency virus and infertility treatment. *Fertil Steril* 2002;77:212–22.

64 Sauer MV. Providing fertility care to those with HIV: time to re-examine healthcare policy. *Am J Bioeth* 2003;3:33–40.

65 Thackway S, Furner V, Mijch A, Cooper DA, Holland D, Martinez P, *et al*. Fertility and reproductive choice in women with HIV-1 infection. *AIDS* 1997;11:663–7. 43

66 Savasi V, Lanzani C, Persico T, *et al*. Pregnancy by self insemination in women infected by HIV-1 (Abstract). 57th Annual Meeting of the
44 American Society for Reproductive Medicine; 2001. *Abstract Book* p349.

67 Hull MG, Glazener CM, Kelly NJ, Conway DI, Foster PA, Hinton RA, *et al*. Population study of causes, treatment and outcome of infertility.
45 *BMJ* 1985;291:1693–7.

68 Irwin KL, Moorman AC, O'Sullivan MJ, Sperling R, Koestler ME, Soto I, *et al*. Influence of human immunodeficiency virus infection on pelvic
46 inflammatory disease. *Obstet Gynecol* 2000;95:525–33.

69 Bukusi EA, Cohen CR, Stevens CE, Sinei S, Reilly M, Grieco V, *et al*. Effects of human immunodeficiency virus 1 infection on microbial origins of pelvic inflammatory disease and on efficacy of ambulatory oral
47 therapy. *Am J Obstet Gynecol* 1999;181:1374–81.

70 Chirgwin K, Feldman J, Muneyyirci-Delale, Landesman S, Minkoff H. Menstrual function in HIV infected women without AIDS. *J Acquir*
48 *Immune Defic Syndr Hum Retrovirol* 1996;12:489–94.

71 Harlow S, Schuman P, Cohen M, Ohmit SE, Cu-Uvin S, Lin X, *et al*. Effect of HIV infection on menstrual cycle length. *J Acquir Immune*
49 *Defic Syndr* 2000;24:68–75.

72 Clark R, Mulligan K, Stamenovic E, Chang B, Watts H, Andersen J, *et al*. Frequency of anovulation and early menopause among women enrolled in selected AIDS Clinical Trials Group studies. *J Infect Dis*
50 2001;184:1325–7.

73 Coll O, Suy A, Vernaeve V, *et al*. Associated factors to the low reproductive outcome in infertile HIV-infected women (Abstract O-022).
51 *Hum Reprod* 2005; (Suppl).

74 Brocklehurst P, French R. The association between HIV infection and perinatal outcome: a systematic review of the literature and meta-analysis. *Br J Obstet Gynaecol* 1998;105:836–48.

75 Frodsham LCG, Cox AD, Almeida PA, Rozis G, Gilling-Smith C. In vitro fertilisation in HIV positive women: risk of mother to embryo viral transmission. *Hum Reprod* 2004;19(Suppl 1):138.

76 Bertrand E. Presence of HIV-1 in follicular fluids, flushes and cumulus oophorus cells of HIV-1-seropositive women during assisted reproduction technology. *AIDS* 2004;18:823–5.

77 Gilling-Smith C, Emiliani S, Almeida P, Liesnard C, Englert Y. Laboratory safety during assisted reproduction in patients with blood-borne viruses. *Hum Reprod* 2005;20:1433–8.

78 Letur-Könirsch H, Collin G, Sifer C, Devaux A, Kuttenn F, Madelenat P, *et al*. Safety of cryopreservation straws for human gametes or embryos: a study with human immunodeficiency virus-1 under cryo-
52 preservation conditions. *Hum Reprod* 2003;18:140–4.

79 Tomlinson M, Sakkas D. Is a review of standard procedures for cryopreservation needed? Safe and effective cryopreservation. Should sperm banks and fertility centres move toward storage in nitrogen vapour? *Hum Reprod* 2000;15:2460–3.

80 The European Parliament and the Council of the European Union. Directive 2004/23.EC of the European Parliament and of the Council
53 of 31 March 2004. *Official*.

81 Frodsham LF, Smith JR, Gilling-Smith C. Assessment of welfare of the child in HIV positive couples. *Hum Reprod* 2004;19:2420–3.

82 Minkoff H, Santoro N. Ethical considerations in the treatment of infertility in women with human immunodeficiency virus infection. *N Engl J Med* 2000;342:1748–50.

83 Sharma S, Gilling-Smith C, Semprini AE, Barton SE, Smith JR. Assisted conception in couples with HIV infection. *Sex Transm Infect* 2003; 79:185–8.

84 The Practice Committee of the American Society for Reproductive Medicine. Hepatitis and reproduction. *Fertil Steril* 2004;82:1754–64.

85 The ESHRE Ethics and Law Task Force. Taskforce 8: ethics of medically assisted fertility treatment for HIV positive men and women. *Hum Reprod* 2004;19:2454–6.

86 Klein J, Pena JE, Thornton MH, Sauer MV. Understanding the motivations, concerns, and desires of human immunodeficiency virus 1-serodiscordant couples wishing to have children through assisted reproduction. *Obstet Gynecol* 2003;101:987–94.

87 Gilling-Smith C. Risking parenthood? Serious viral illness, parenting and welfare of the child. In: Shenfield F, Sureau C, editors. *Contemporary Ethical Dilemmas in Assisted Reproduction*, Vol 5. 2006. p. 57–69
54 (in press).

Author Query Form

Journal: An International Journal of Obstetrics & Gynaecology

Article : bjo_960

Dear Author,

During the copy-editing of your paper, the following queries arose. Please respond to these by marking up your proofs with the necessary changes/additions. Please write your answers on the query sheet if there is insufficient space on the page proofs. Please write clearly and follow the conventions shown on the attached corrections sheet. If returning the proof by fax do not write too close to the paper's edge. Please remember that illegible mark-ups may delay publication. Many thanks for your assistance.

Query No.	Query	Remark
1	Author: Please provide the department/unit name for the second affiliation.	
2	Author: Please note that 'HBV and HCV' in the sentence 'Following the Human …' has been spelt out as 'hepatitis B virus and hepatitis C virus', respectively. Please check if this is correct.	
3	Author: Please check whether the expansion for 'NASBA' in the sentence 'A nucleic acid …' should be 'nucleic acid sequence-based amplification'.	
4	Author: Please note that HCG in the sentence 'In a similar fashion …' has been spelt out as 'human chorionic gonadotrophin'. Please check if this is correct.	
5	Author: Please spell out 'VL' in the sentence 'CPR was significantly higher …'	
6	Author: Please check whether 'CREATHE' in the sentence 'The combined database …' should be 'CREATE' as in the previous occurrence.	
7	Author: Details given in reference 28 and 88 were same. Hence, reference 88 has been deleted and the reference citation in text has been renumbered accordingly.	
8	Author: In reference 1, please provide the publisher name and location details (city, state [if USA] and country).	
9	Author: As per style if a reference has more than 6 authors, names of first 6 authors must be listed followed by et al. Hence, in reference 2, names of fourth to sixth author have been included. Please check if this is correct. Also 'R' has been introduced to the initials of the first author, as per PubMed. Please check if this is correct.	
10	Author: As et al. can be used only when a reference has more than six authors, the names of the fourth to sixth author have been introduced in reference 4. Please check if this is correct.	
11	Author: As per style if a reference has more than 6 authors, names of first 6 authors must be listed followed by et al. Hence, in reference 6, names of fourth to sixth author have been included. Please check if this is correct.	

12	Author: Please update reference 11.
13	Author: Please note that 'dvise' has been deleted from article title in reference 13, as per PubMed. Please check if this is correct.
14	Author: As per style if a reference has more than 6 authors, names of first 6 authors must be listed followed by et al. Hence, in reference 15, names of fourth to sixth author have been included. Please check if this is correct.
15	Author: As per style if a reference has more than 6 authors, names of first 6 authors must be listed followed by et al. Hence, in reference 16, names of fourth to sixth author have been included. Please check if this is correct.
16	Author: As per style if a reference has more than 6 authors, names of first 6 authors must be listed followed by et al. Hence, in reference 17, names of fourth to sixth author have been included. Please check if this is correct.
17	Author: In reference 19, please provide the date and month; location of the meeting; publisher name and location and the year.
18	Author: As per style if a reference has more than 6 authors, names of first 6 authors must be listed followed by et al. Hence, in reference 20, names of fourth to sixth author have been included. Please check if this is correct.
19	Author: As per style if a reference has more than 6 authors, names of first 6 authors must be listed followed by et al. Hence, in reference 21, names of fourth to sixth author have been included. Please check if this is correct.
20	Author: As et al. can be used only when a reference has more than six authors, the names of the fourth to sixth author have been introduced in reference 22. Please check if this is correct.
21	Author: As per style if a reference has more than 6 authors, names of first 6 authors must be listed followed by et al. Hence, in reference 25, names of fourth to sixth author have been included. Please check if this is correct.
22	Author: As per style if a reference has more than 6 authors, names of first 6 authors must be listed followed by et al. Hence, in reference 26, names of fourth to sixth author have been included. Please check if this is correct.
23	Author: As per style if a reference has more than 6 authors, names of first 6 authors must be listed followed by et al. Hence, in reference 30, names of fourth to sixth author have been included. Please check if this is correct.
24	Author: As per style if a reference has more than 6 authors, names of first 6 authors must be listed followed by et al. Hence, in reference 31, names of fourth to sixth author have been included. Please check if this is correct.
25	Author: As et al. can be used only when a reference has more than six authors, the names of the fourth to sixth author have been introduced in reference 32. Please check if this is correct.
26	Author: As per style if a reference has more than 6 authors, names of first 6 authors must be listed followed by et al. Hence, in reference 34, names of fourth to sixth author have been included. Please check if this is correct.
27	Author: In reference 35, please provide the date, month and year; location of the symposium; publisher name and location and year.

28	Author: If reference 38 has more than 6 authors, then list the names of first 6 authors followed by et al.; otherwise, provide the names of all the authors.
29	Author: If reference 39 has more than 6 authors, then list the names of first 6 authors followed by et al.; otherwise, provide the names of all the authors.
30	Author: As et al. can be used only when a reference has more than six authors, the names of the fourth to sixth author have been introduced in reference 40. Please check if this is correct.
31	Author: As per style if a reference has more than 6 authors, names of first 6 authors must be listed followed by et al. Hence, in reference 41, names of fourth to sixth author have been included. Please check if this is correct.
32	Author: In reference 42, please check what 'S1' indicates. Also, please check the page range.
33	Author: As per style if a reference has more than 6 authors, names of first 6 authors must be listed followed by et al. Hence, in reference 44, names of fourth to sixth author have been included. Please check if this is correct.
34	Author: In reference 46, the spelling of 'intracytoplasmatic' has been changed to 'intracytoplasmic'. Please check if this is correct.
35	Author: Please provide the page range for reference 49.
36	Author: If reference 50 has more than 6 authors, then list the names of first 6 authors followed by et al.; otherwise, provide the names of all the authors.
37	Author: If reference 51 has more than 6 authors, then list the names of first 6 authors followed by et al.; otherwise, provide the names of all the authors.
38	Author: If reference 53 has more than 6 authors, then list the names of first 6 authors followed by et al.; otherwise, provide the names of all the authors.
39	Author: As per style if a reference has more than 6 authors, names of first 6 authors must be listed followed by et al. Hence, in reference 57, names of fourth to sixth author have been included. Please check if this is correct.
40	Author: Please provide the publisher name in reference 58.
41	Author: As per style if a reference has more than 6 authors, names of first 6 authors must be listed followed by et al. Hence, in reference 60, names of fourth to sixth author have been included. Please check if this is correct.
42	Author: As et al. can be used only when a reference has more than six authors, the names of the fourth to sixth author have been introduced in reference 61. Please check if this is correct.
43	Author: As per style if a reference has more than 6 authors, names of first 6 authors must be listed followed by et al. Hence, in reference 65, names of fourth to sixth author have been included. Please check if this is correct.
44	Author: Please provide the date and month; location of the meeting; publisher name and location and year in reference 66. Also, please check whether 'Abstract Book p349' can be deleted from the reference.

45	Author: As per style if a reference has more than 6 authors, names of first 6 authors must be listed followed by et al. Hence, in reference 67, names of fourth to sixth author have been included. Please check if this is correct. Also, please check if the introduced initials in the first, second, third and fourth author is correct.
46	Author: As per style if a reference has more than 6 authors, names of first 6 authors must be listed followed by et al. Hence, in reference 68, names of fourth to sixth author have been included. Please check if this is correct. Also, please check if the introduced initials in the first, second and third author is correct.
47	Author: As per style if a reference has more than 6 authors, names of first 6 authors must be listed followed by et al. Hence, in reference 69, names of fourth to sixth author have been included. Please check if this is correct. Also, please check if the introduced initials in the first, second and third author is correct.
48	Author: As et al. can be used only when a reference has more than six authors, the names of the fourth to sixth author have been introduced in reference 70. Please check if this is correct.
49	Author: As per style if a reference has more than 6 authors, names of first 6 authors must be listed followed by et al. Hence, in reference 71, names of fourth to sixth author have been included. Please check if this is correct.
50	Author: As per style if a reference has more than 6 authors, names of first 6 authors must be listed followed by et al. Hence, in reference 72, names of fourth to sixth author have been included. Please check if this is correct.
51	Author: If reference 73 has more than 6 authors, then list the names of first 6 authors followed by et al.; otherwise, provide the names of all the authors. Also, please provide the volume number and page range.
52	Author: As per style if a reference has more than 6 authors, names of first 6 authors must be listed followed by et al. Hence, in reference 78, names of fourth to sixth author have been included. Please check if this is correct.
53	Author: Please note that reference 80 seems to be incomplete. Please check. If so, please provide the necessary details.
54	Author: Please provide the publisher name and location for reference 87 and also update the reference.
55	Author: In Tables 1–3, please note that 'OPR' has been spelt out as 'outgoing pregnancy rate' in the footnote. Please check if this is correct. Also, please note that as per journal style, use of the word 'ongoing' is not preferred, instead, it should be replaced with 'continuing'. Hence, please make the appropriate change to the words 'ongoing pregnancy' and 'ongoing pregnancy rate' in Tables 1–3 and also to the abbreviation used for the same both in table body and in table footnote.
56	Author: Please spell out 'N/A' in Table 2 footnote. Please check whether it can be represented as 'NA'.

MARKED PROOF

Please correct and return this set

Please use the proof correction marks shown below for all alterations and corrections. If you wish to return your proof by fax you should ensure that all amendments are written clearly in dark ink and are made well within the page margins.

Instruction to printer	*Textual mark*	*Marginal mark*
Leave unchanged	··· under matter to remain	Stet
Insert in text the matter indicated in the margin		New matter followed by
Delete	through matter to be deleted	
Delete and close up	through matter to be deleted	
Substitute character or substitute part of one or more word(s)	/ through letter or through word	New letter or new word
Change to italics	— under matter to be changed	
Change to capitals	≡ under matter to be changed	
Change to small capitals	= under matter to be changed	
Change to bold type	~ under matter to be changed	
Change to bold italic	≈ under matter to be changed	
Change to lower case	Encircle matter to be changed	
Change italic to upright type	(As above)	
Insert 'superior' character	/ through character or where required	under character e.g.
Insert 'inferior' character	(As above)	over character e.g.
Insert full stop	(As above)	
Insert comma	(As above)	
Insert single quotation marks	(As above)	and/or
Insert double quotation marks	(As above)	and/or
Insert hyphen	(As above)	
Start new paragraph		
No new paragraph		
Transpose		
Close up	linking letters	
Insert space between letters	between letters affected	
Insert space between words	between words affected	
Reduce space between letters	between letters affected	
Reduce space between words	between words affected	