

# Mx2 expression is associated with reduced susceptibility to HIV infection in highly exposed HIV seronegative Kenyan sex workers

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**Introduction:** Recent studies have identified Mx2 as a novel HIV-1 innate restriction factor that inhibits proviral integration. A pilot proteomic study of immune cells from highly exposed HIV-seronegative (HESN) individuals enrolled in the Pumwani sex worker cohort identified Mx1 as potential correlate of HIV protection. A detailed population level analysis of Mx1 and Mx2 expression and their role in reduced susceptibility to HIV infection in HESN women was conducted.

**Methods:** Peripheral blood mononuclear cells (PBMC) were isolated from 102 HESN women and 100 high-risk negative controls enrolled in a Nairobi-based sex worker cohort. Whole-cell lysates were prepared and analyzed for Mx1 and Mx2 expression by commercial ELISA. Bivariate and multiple linear regression analyses were conducted to account for confounding epidemiological factors.

**Results:** Mx2, but not Mx1, was found to be significantly overexpressed in HESN women compared with high-risk negative controls ( $P=0.027$ ). After multiple linear regression analysis, accounting for age, menopause, pregnancy, Depo-Provera (Pfizer, New York, USA) use, recent infections and medication usage, Mx2 expression remained significantly overexpressed in the PBMC of HESN women ( $P=0.05$ ). Additionally, an interaction model analysis indicated that HESN women who use Depo-Provera (Pfizer, New York, USA) have 2.6-fold higher levels of Mx2 than any other group ( $P<0.001$ ). No associations with Mx1 expression were observed.

**Conclusion:** This is the first epidemiological report of Mx2 and its association with altered susceptibility to HIV infection in HESN women. Additionally, we show that HESN women who use Depo-Provera (Pfizer, New York, USA) have the highest levels of Mx2 expression, highlighting a possible mechanism for hormonal modulation of HIV susceptibility.

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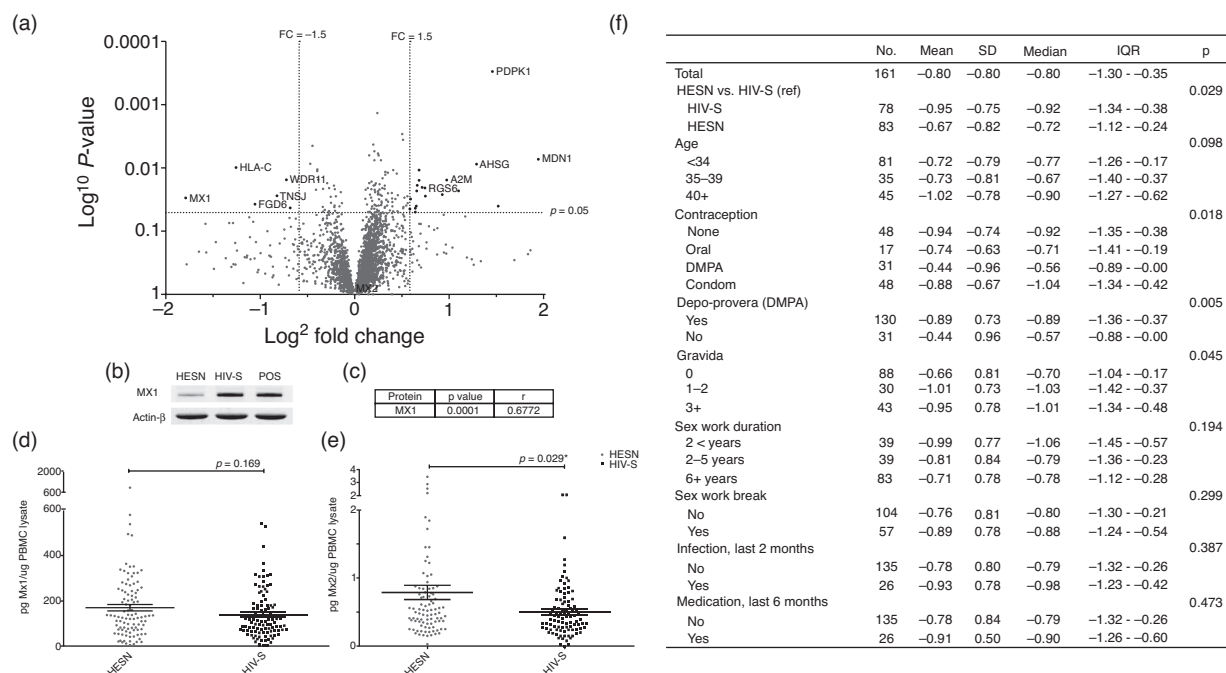
## Introduction

We have previously described a cohort of highly exposed HIV-seronegative (HESN) sex workers from Nairobi, Kenya, who can be epidemiologically defined as relatively resistant to HIV infection [1]. The phenotype of these HESN women has been shown to associate with altered interferon signaling mediated in part by interferon regulatory transcription factor-1 [2], T-cell immune quiescence [3], and using unbiased proteomic techniques, the altered expression of antiproteases in genital secretions [4,5]. However, a proteomics analysis of mucosal and systemic immune cells in the context of this rare phenotype has yet to be undertaken. In a pilot proteomics study of peripheral blood mononuclear cells (PBMC) isolated from HESN ( $n=12$ ) and HIV-susceptible controls ( $n=18$ ), we identified decreased expression of Mx1 ( $P=0.029$ ) as a potential marker of HIV susceptibility in HESN women (Fig. 1a). Validation of Mx1 by western blot and correlation analysis ( $r=0.6772$ ,  $P=0.0001$ ) confirmed the lower Mx1 expression in the same HESN samples (Fig. 1b and c). The reduced expression of Mx1, and possibly other members of this family in HESN women, may reflect the altered interferon responsiveness previously observed in these individuals [6]. Additionally, recent studies have shown that Mx2 is a correlate of simian immunodeficiency virus (SIV) protection in macaques [7], restricts HIV-1

infection *in vitro* [8–10], and is likely due to the destabilization of viral replication complexes or nuclear import [11]. Although Mx2 was not differentially regulated in our initial pilot data (Fig. 1a), these recent studies and the similar amino acid homology between the two proteins (~63%), led us to conduct a more detailed population level analysis of Mx1 and Mx2 expression in HESN women from the Pumwani sex worker cohort.

## Results

To determine the expression levels of Mx1 and Mx2 in a larger study accounting for epidemiological covariates, we isolated PBMC from 102 HESN women meeting our epidemiologic definition of HIV resistance and 100 high-risk HIV-susceptible female sex workers (HIV-S). HESN women are epidemiologically defined as being active in sex work while remaining HIV negative (serology and PCR) for 7 years or longer. Given the volume of clients, the estimated prevalence of HIV in this population, and duration of seronegative follow-up, it is clear these individuals have intense exposure to HIV. A commercial ELISA was used to measure the levels of Mx1 and Mx2 in PBMC lysate from the two study groups. Bivariate comparisons indicated that the Mx1 expression in PBMC was similar between HESN women and HIV-S controls (Fig. 1d,  $P=0.169$ ), clearly in disagreement with our



**Fig. 1. Expression of Mx1 and Mx2 in highly exposed HIV-seronegative women.** (a) Protein biomarkers of interest differentially regulated between highly exposed HIV-seronegative (HESN) and HIV-susceptible controls ( $P < 0.05$  and a FC  $> 1.5$ ). (b) Representative western blot analysis at the patient level with an HIV-infected individual included for comparative purposes and (c) correlation between the mass spectrometry and western blot expression. Expression levels are illustrated for (d) Mx1 and (e) Mx2 measured by commercial ELISA in 102 HESN and 100 HIV-susceptible controls. (f) Bivariate analysis of epidemiological factors with respect to log Mx2 expression, excluding pregnant and postmenopausal women.

proteomics pilot study. These discrepancies highlight the issues associated with accurately controlling for epidemiological confounders in proteomics or other systems biology approaches that are often found in small-scale human studies. Surprisingly, in contrast, Mx2 expression was found to be elevated in HESN women ( $P=0.029$ ) compared to HIV-S controls (Fig. 1e). Univariate analysis of epidemiologic confounders in these women showed factors such as age, duration of sex work, a break from sex work, and recent infections or medication use did not affect the expression of Mx2 (Fig. 1f) or Mx1 (Suppl. Table 1, <http://links.lww.com/QAD/A590>). However, when we examined contraception use, we found that women who reported using injectable Depo-Provera (DMPA; Pfizer, New York, USA) had significantly higher expression of Mx2 (Fig. 1f;  $P=0.005$ ) compared with those who did not use DMPA.

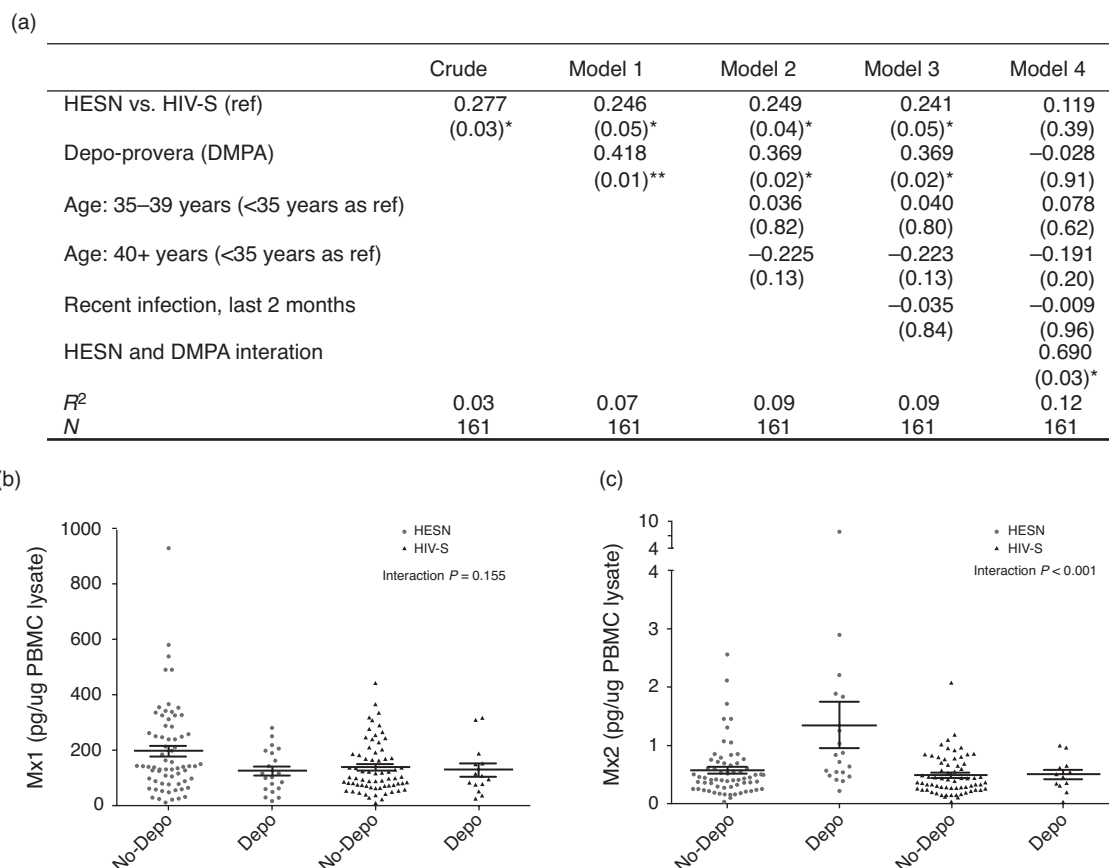
To better define the relationship between DMPA use and systemic cellular expression of Mx1 and Mx2, multiple linear regression analysis was conducted to account for epidemiological confounders. In a multiple linear regression model adjusted for DMPA use, age, and current infections, the increased Mx2 expression

associated with HESN women remained significant (Fig. 2a;  $P=0.05$ ; model 3). An interaction indicator model between DMPA use and HESN status was also tested (Fig. 2a, model 4), and found that HESN women who use DMPA accounted for the majority of increased Mx2 expression observed in this group. The mean Mx2 expression in the PBMC of HESN women reporting DMPA use was on average 2.6-fold higher (1.35  $\mu\text{g}/\mu\text{g}$  of PBMC lysate) than any other group (Fig. 2c;  $P < 0.0001$ ). Using the same linear regression analysis and interaction model no significant relationships for Mx1 were noted (Fig. 2b & Suppl. Table 2, <http://links.lww.com/QAD/A590>). Together these findings suggest an association between the HESN phenotype, DMPA use, and Mx2, but not Mx1 expression.

## Methods

### Cohort participants

The Pumwani sex worker cohort is an open cohort that provides HIV-1 prevention services and treatment through funding from the Presidents Emergency Plan



**Fig. 2. DMPA use and its effect on Mx1/Mx2 expression in highly exposed HIV-seronegative women.** (a) Linear regression modeling accounting for DMPA, age, recent infection, and the DMPA interaction with respect to Mx2 expression in HESN women compared with HIV-susceptible controls. The expression of Mx1 (b) and Mx2 (c) in highly exposed HIV-seronegative (HESN) and HIV-susceptible women with reported DMPA use. \* $P < 0.05$ , \*\* $P \leq 0.01$ .

For AIDS Relief. The cohort has enrolled over more than 3800 female sex workers, with approximately 250 new enrolments per year. These women are at extremely high risk of HIV-1 infection, with 50% of women being HIV-1 seropositive at enrollment. This cohort includes 1285 women in active follow-up, with 671 infected and 614 uninfected, of which 107 HESN participants meet our epidemiologic definition of resistance to HIV (HIV-R) infection (>7 years in the cohort, active in sex work, and HIV-1 negative by ELISA and DNA/RNA testing). Women who are newly enrolled in the cohort, with 3 years or less of follow-up (HIV-S) are considered to be at the same risk of acquiring HIV infection as HIV-R women and act as an HIV-S control. Based upon 27 years of epidemiologic follow-up, we expect 85% of the HIV-S to seroconvert, despite strong HIV-1 prevention programmes. Both HIV-R and HIV-S have similar numbers of clients per day, as well as reported condom use with casual and regular clients. Written informed consent was obtained from all study participants of the Pumwani cohort. Approval for all studies was obtained from the research ethics board of the University of Manitoba, Winnipeg, Canada and the Kenyatta National Hospital, Nairobi, Kenya.

### Sample preparation

PBMC were isolated for the pilot mass spectrometry and epidemiological studies by density gradient centrifugation (Ficoll-Paque). PBMC samples for the epidemiological studies conducted on Mx1 and Mx2 in the Pumwani cohort were isolated in Nairobi, Kenya, from 1995 to 2009. Sample storage length was found not to have any effect on Mx1/2 protein expression as well as cell viability by correlation analysis. PBMC were frozen in freezing media and shipped back to Winnipeg, Manitoba, Canada, and stored in liquid nitrogen. PBMCs were removed from the liquid nitrogen tank and rapidly thawed in a 37°C water bath. Cells were transferred from the cryovial and immediately placed in 10 ml of RPMI supplemented with 10% fetal calf serum, penicillin, streptomycin, and fungizone (Life Technologies, Carlsbad, California, USA). Cell viability and counts were conducted on each sample to ensure a viability of at least 80% using the Invitrogen Countess Automated Cell Counter (Carlsbad, California, USA) by trypan blue staining. After counting, the cells were centrifuged at 800 g for 10 min. Cells were washed in PBS three times. Cells were then resuspended in either 200 µl of 4% SDS (MS analysis) or 500 µl of PBS (ELISA analysis). PBMCs suspended in PBS were subjected to three rapid freeze thaw cycles on dry ice. The resulting PBMC lysate was centrifuged at 1500 g to remove any excess cellular debris. PBMCs suspended in 4% SDS were immediately boiled for 5 min followed by centrifugation at 1500 g to remove any excess cellular debris. PBMC lysate was transferred to a new cryovial and stored at -80°C for future use by mass spectrometry or ELISA analysis. The total protein concentration of PBMC lysate in 4% SDS and PBS

was determined by 2D-Quant (GE Healthcare, Chalfont, UK) and BCA assay (Millipore, Billerica, Massachusetts, USA), respectively.

### Isobaric tags for relative and absolute quantitation mass spectrometry

Trypsin digestion of PBMC lysate in 4% SDS was performed by the filter-aided sample preparation method [12] with modifications, briefly described as follows: approximately 500 µg of protein per digestion was mixed with 7× its volume in urea exchange buffer, then passed through a 10 kDa Nanosep spin filter (Pall Corp., Port Washington, New York, USA). Two additional washes with UEB were performed to remove excess SDS. Protein was then alkylated with 50 mmol/l iodoacetamide for 20 min at room temperature in the dark. Filters were washed twice with UEB, treated with 1000 units of benzonase (Novagen, Madison, Wisconsin, USA) in 50 mmol/l Tris or 50 mmol/l HEPES for 30 min at room temperature, then rinsed again with Tris or HEPES buffer. Each filter was treated with 1.5 µg of trypsin gold (Promega, Madison, Wisconsin, USA) per 100 µg protein overnight at 37°C. Filter cartridges were inverted and the peptides were spun off the filter with three washes of 50 mmol/l Tris or HEPES. Peptides were lyophilized, resuspended in water, and quantified by ultraviolet absorbance at 280 nm. Samples were then frozen at -80°C for future analysis or processing. When isobaric tags for relative and absolute quantitation (iTRAQ) labeling was required, digested peptides were resuspended in 30 µl of 100 mmol/l HEPES. The contents of a single iTRAQ reagent tube (AB Sciex, Framingham, Massachusetts, USA) were resuspended in 70 µl of ethanol and added to the peptides. The pH of each reaction was verified and adjusted to pH 8 if necessary for optimal labeling efficiency. After labeling, 100 µl of H<sub>2</sub>O was added to quench any further labeling.

In order to ensure equal, one-to-one ratios between all iTRAQ reporter ions and verification of efficient labeling, equal volumes (1 µl) of each labeled sample were mixed with buffer A (2% acetonitrile, 0.1% formic acid) to a final volume of 20 µl, and analyzed by online nLC/MS/MS (see below). Ratios from the most abundant proteins were then used to make minor corrections to the final mixing volumes ensuring equal 1:1 ratios for all reporter channels.

Samples were multiplexed to ensure an overall 1:1 ratio for all the iTRAQ reporter channels. 75 µg of peptide was lyophilized and resuspended in 200 mmol/l ammonium formate, pH 10 and loaded onto an offline high-pH reversed phase column for fractionation (Waters, Milford, Massachusetts, USA; XBridge C18 3.5 µm 2.1×100 mm) described previously [13]. Samples were fractionated using an Agilent 1200 series microflow pump (Agilent, Santa Clara, California, USA) with buffer A (20 mmol/l ammonium formate, pH 10) and buffer B

(90% ACN, 20 mmol/l ammonium formate, pH 10). The samples from each patient were then generally subjected to one of two linear gradients. A non-iTRAQ labeled gradient (0–60% buffer B at 150  $\mu$ l/min for 66 min) or a iTRAQ specific gradient (3% B – 10 min, 8–11.5% B – 7 min, 11.5–60% B – 58 min, at 150  $\mu$ l/min). A total of 24 fractions were collected over the peptide elution profile and then concatenated into 12 final fractions. Fractions were then lyophilized and stored at  $-80^{\circ}\text{C}$  for future MS analysis.

Peptide fractions were resuspended in nano-LC buffer A (0.1% formic acid, 2% ACN) and approximately 1.5  $\mu$ g from each fraction was injected onto a 2-cm precolumn (id = 100- $\mu$ m) consisting of 5- $\mu$ m particle-sized ReproSil-Pur C18- AQ resin (Dr Maisch GmbH, Beim Bruckle, Germany). Peptides were further resolved on a 10-cm analytical column (id = 75- $\mu$ m) consisting of 3- $\mu$ m particle-sized ReproSil-Pur C18-AQ resin (Dr Maisch GmbH) using an Easy-nLC system (Thermo Fisher Scientific, Waltham, Massachusetts, USA) with a linear gradient from 0 to 40% buffer B (0.1% formic acid, 98% ACN) over 120 min at a flow rate of 300 nl/min. The total runtime was 160 min, which included sample loading, gradient elution, ACN wash, and column equilibration. Mass spectra were acquired using a data-dependent method using an Orbitrap Velos mass spectrometer (Thermo Fisher Scientific). The top 10 abundant precursor ions from each survey scan were isolated (2.0 m/z isolation width) and fragmented by higher-energy collision dissociation (45% normalized collision energy, with 100 ms activation time). The survey scans, were acquired in the Orbitrap using a mass window of 300–1700 m/z at a target resolution of 60 000, at 400 m/z, and the subsequent fragment ion scans were acquired over a dynamic m/z range. The lower threshold for selecting a precursor ion for fragmentation was 1000 ions. Dynamic exclusion was enabled using a list size of 500 features, an m/z tolerance of 15 ppm, a repeat count of 1, a repeat duration of 30 s, and an exclusion duration of 15 s, with early expiration disabled.

RAW (Thermo Fisher) data files from the Orbitrap Velos were processed using the Mascot search engine (v2.3 Matrix Science, London, UK). All data were searched against the International Protein Index (IPI version 3.78; Ingenuity IPA, Redwood City, California, USA) human database. The following search parameters were used: monoisotopic precursor tolerance was set to 10 ppm while the MS/MS tolerance was set to 0.5 Da. Enzyme specificity was set to trypsin with a maximum of one missed cleavage. Carbamidomethyl of cysteine, iTRAQ labeling of N-termini and lysine's were all applied as fixed modifications. Oxidation of methionine, and iTRAQ labeling of tyrosine were set as variable modifications. All donors were imported into an instance of Trans Proteomic Pipeline implemented in Scaffold v3.4.9 (Proteome Software, Portland, Oregon, USA).

X!Tandem (thegpm.org) was used within Scaffold as an additional measure for confident peptide and protein identifications (thegpm.org; version CYCLONE, 2010.12.01.1). Quantitative results were exported from Scaffold with the following criteria: minimum peptide probability of 80% (Peptide Prophet; Protein & Peptide Prophet, Seattle, Washington, USA) a minimum protein probability of 99% (Protein Prophet), and a minimum of two peptides per protein. These setting resulted in a 0.1% protein and 1.5% peptide false discovery rate for the entire dataset. Statistical significance between the respective study groups was evaluated using a Student's *t*-test for each protein comparison.

### Western blot analysis

PBMC lysate (25  $\mu$ g) was loaded on to a NuPAGE 4–12% Bis-Tris precast acrylamide gel (Life Technologies). Affinity MagicMark™ XP (Life Technologies) was also loaded as molecular weight marker. Gels were resolved to completion using an XCell SureLock Mini-Cell (Invitrogen, Carlsbad, California, USA) electrophoresis tank running at 150 V. After protein gel electrophoresis, proteins were transferred onto a nitrocellulose membrane using the Invitrogen iBlot (Invitrogen) transfer system. Membranes were directly placed in Odyssey blocking buffer (OBB; LI-CORE) for 1 h at room temperature with gentle mixing. Primary antibodies Mx1, RGS6, and HLA-C (Epitomics) were diluted in OBB with the addition of 0.1% Tween-20 (Sigma) and incubated overnight at  $4^{\circ}\text{C}$  on an orbital shaker. An antibody loading control ( $\beta$ -Actin; Abcam) incubated for 1 h at room temperature followed by four 5-min washes with phosphate-buffered saline–Tween (PBS, 0.1% Tween-20). Secondary fluorescent antibodies (IRDye 680R/800CW, diluted in OBB, 0.1% Tween 20, and 0.01% SDS; LI-CORE) were then incubated at room temperature for 1 h in the dark followed by another four 5-min washes with PBST. A final wash of PBS for 5 min was performed. Blots were subsequently dried at  $4^{\circ}\text{C}$  and imaged using an Odyssey CLx infrared imaging system (LI-CORE).

### Mx1 and Mx2 ELISA

A commercial ELISA was purchased from USCN Life Science Inc. (Wuhan, China) for the quantification of Mx1 and Mx2 in PBMC lysate. Briefly, a standard doubling dilution series was prepared for either Mx1 or Mx2 (20–0.02 ng/ml) and PBMC lysate samples in PBS from each study participant were added to the plates in duplicate. The plates were sealed and incubated for 2 h at  $37^{\circ}\text{C}$ . After incubation the samples and standard curve were aspirated from the plate using the Biotek plate washer. A biotin-conjugated antibody specific for Mx1 or Mx2 was added (1 : 100 dilution) followed by incubation at  $37^{\circ}\text{C}$  for 60 min. After incubation the plates were manually washed three times with the wash buffer provided in the ELISA kit. A secondary avidin conjugated

to horseradish peroxidase was then added (1:100 dilution) and incubated for 30 min at 37°C. The plates were again washed five times with the provided wash buffer. Tetramethylbenzidine substrate was then added to the plates and incubated at 37°C for 15–30 min. Stop solution (sulphuric acid) was then added to terminate the reaction. The plates were read using the SpectraMax Plus spectrophotometer at a wavelength of 450 nm. A four-parameter curve was used to fit the standard curve for accurate quantitation of Mx1 and Mx2.

### Epidemiological analysis

The Mx1 and Mx2 expression levels measured by the commercial ELISA were transformed using their natural logarithms. The log-transformed values of Mx1 and Mx2 were subsequently used as outcome variables in separate multiple linear regression models. Where appropriate, *t*-tests and ANOVA's were used in bivariate analyses with logged values of Mx1/Mx2 used as the outcome variables. Multiple linear regression models were fitted to the data and used to assess the independent association of HESN status and the logged values of Mx1 and Mx2, adjusted for age, recent infections (2 months) and Depo-Provera. Given their theoretical importance, all confounders were kept in full linear regression models; however, each confounder was entered separately in the following order: use of Depo-Provera, age, and recent infection in the last 2 months. In addition, the interaction between Depo-Provera use and HESN status was added as an additional variable in the final full model. All tests performed were considered statistically significant at  $P < 0.05$ . Stata 12 (College Station, Texas, USA) was used for all epidemiological analyses. Age, recent infections, and Depo-Provera were included a priori due to the impact of these variables on general immune function. The interaction between HESN status and Depo-Provera was also planned a priori due to our interest in whether or not levels of Mx1/Mx2 between HESN and high-risk negative controls differed by use of Depo-Provera. From an initial sample of 202 women, 190 had detectable levels for both Mx1 and Mx2. For all models, menopausal ( $n = 2$ ), pregnant ( $n = 5$ ) and missing values for contraception use ( $n = 22$ ) were excluded from the analyses, leaving a total sample size of 161 women.

### Discussion

Although still controversial [14], several studies have linked DMPA use with increased risk of HIV acquisition [15,16]. These studies suggest that HESN women using DMPA are likely at a higher risk of infection than HESN women who do not use DMPA. The high levels of Mx2 observed in HESN women, epidemiologically resistant to HIV, may provide a plausible biologic mechanism to explain how this particular subset of HESN women is protected from infection. DMPA is commonly used in sub-Saharan Africa and represents a relatively inexpensive

alternative to oral or intrauterine device contraception. For this reason, it is possible that DMPA use could be confounded by some other epidemiological factor not captured in our study. However, given that DMPA has been shown to suppress interferon responses, and that interferon-stimulated genes, such as Mx2, have been associated with protection from SIV infection in rhesus macaques, a biological interaction between DMPA and Mx2 in HESN women is plausible [17,18]. Although it is tempting to suggest that DMPA alone could be used to modulate resistance, its broad immunosuppressive effects and reported increase in HIV acquisition risk will most likely eliminate its use in preventive strategies. However, identification of an intermediate biologic link between DMPA and innate Mx2 signaling in HESN women may allow for targeted regulation of Mx2 as a novel prevention strategy. Interventions such as RNA interference of known regulators of Mx2 or through as yet defined viral/host mRNA mechanisms could alternatively be used to regulate HIV resistance and susceptibility via Mx2. Data from our group and others have shown that the HESN phenotype is multifactorial; however, one commonality is the altered expression of innate factors limiting HIV replication, immune activation, and the recruitment of target cells [3]. Increased expression of Mx2 in HESN women using DMPA may suggest a link to this immune quiescent phenotype; however, a causal relationship between altered interferon signaling and the genetic regulation of Mx2, influenced by hormonal contraception, needs to be established. One recent study has suggested that Mx2 expression of injection drug users and sexually exposed HIV individuals may play a role in altered susceptibility to HIV infection [19]. Thus, further studies of the relationship between HIV susceptibility, Mx2 expression, and DMPA use are clearly warranted. These data represent the first report of Mx2 as a contributor to altered susceptibility to HIV infection in HESN female sex workers in the context of epidemiologic confounders such as hormonal contraception use. Future studies need to identify mechanisms that may regulate higher expression of Mx2 in HESN women and its interaction with DMPA. A deeper understanding of interferon responses to HIV in the real-world setting could lead to HIV preventative measures that make use of Mx2 to modulate resistance and susceptibility to HIV infection.

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Author contributions: D.R.S., K.R.F., F.A.P., and T.B.B. designed the study and wrote the manuscript. D.R.S. carried out the experiments. Sample preparation and technical expertise was provided by M.A., G.R.W. and S.J.M. provided mass spectrometry expertise and equipment. S.Y.S. and L.R.M. facilitated the epidemiological analysis. All authors read and approved the final manuscript.

### Conflicts of interest

There are no conflicts of interest.

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