

Supplementary File

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Box S1: Specimen processing and testing for chemo-cytokines, HIV viral load and *T. pallidum* PCR and serological tests

Blood was drawn into vacuum EDTA-coated and serum gel tubes (Sarstedt Monovette, Nümbrecht, Germany). Plasma was separated and stored at -80°C within three hours of sampling. All samples were processed in the same fashion according to the study standard operating procedures and were only subjected to one freeze-thaw cycle.

Cytokines (Interferon [IFN] α , IFN γ , Interleukin [IL]-1 β , IL-12p40, IL-12p70, IL-4, IL-5, IL-6, IL-7, IL-10 and IL-17A and chemokines (IFN γ -Inducible Protein [IP]-10, Monocyte Chemoattractant Protein [MCP]-1, Macrophage Inflammatory Protein [MIP]-1 α , MIP-1 β , and IL-8) from the baseline and 6-month time-points were measured in a single experiment using a magnetic bead Milliplex™ Human Cytokine kit (EMD Millipore, Billerica, MA, USA) on a Bio-Plex™ Suspension Array Reader (Bio-Rad Laboratories Inc, Hercules, CA, USA) according to manufacturer's instructions. Samples below the limit of quantification were assigned the value of half the lowest limit detected for each cytokine (Supplementary Table S1). Standard curves were constructed using duplicate measurements of kit standards.

HIV viral loads below the limit of detection (20 copies/mL) were given the value of 10 copies/mL. In case of clinical suspicion of concomitant sexually transmitted infections (STIs) the presence of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* was assessed via an Abbott RealTime CT/NG assay (Abbott Molecular, Des Plaines, IL, USA).

***T. pallidum* PCR and serological testing**

Treponema pallidum DNA was detected in serum, plasma and in ulcer material, if ulcer was present, by means of an in-house real time PCR targeting *poIA*. The ulcer material was collected using a flocced synthetic swab (COPAN innovation, Italy) and eluted in 1.2 ml diluted phosphate buffered saline (PBS) (1,200 μ L; 1 part PBS and 9 parts saline, pH 7.4). In short, DNA was extracted from 400 μ L plasma and serum and from 400 μ L of the eluted ulcer material, using the custom-plasma program of the Abbott m2000sp automated extraction platform (Abbott, Maidenhead, UK), according to the manufacturer's specifications. The extracted DNA was eluted in 100 μ L eluate volume. The 25 μ L PCR mixture contained Platinum® Quantitative PCR SuperMix-UDG (Invitrogen by life technologies, Thermo Fisher Scientific) 0,9 μ M forward primer TP-1, 0,9 μ M reverse primer TP-2, 0,14 μ M probe TP-3 (Integrated DNA technologies, Leuven, Belgium) [1] and 10 μ L DNA extract. The amplification were performed with the Rotor Gene 6000 (Qiagen, Venlo, the Netherlands) and included 45 cycles of 20 sec at 95 °C followed by 45 sec at 60 °C per cycle.

Syphilis serological testing was performed on serum with the Macro-Vue RPR card (Becton, Dickinson and Company, Sparks, MD, USA) and the *T. pallidum* particle agglutination (TPPA) SERODIA-TPPA (Fujirebio Inc., Tokyo, Japan) tests.

Table S1. Cytokine and chemokine data quality assessment.

Cytokine	CV values of duplicate samples with detectible values (N=detectable sample(s)/ CV%)	Correlation of subset (N=15) of samples tested in duplicate [§]	P-value	Samples with undetectable concentrations (%)	Lowest level of detection (pg/mL)
IFN α	5/ 37.9	0.81	0.0005	49.5	1.6
IL1 β	#	0.57	0.0342	96.3	3.1
IL-6	#	##	-	92.2	2.5
IL-17A	5/ 14.7	0.67	0.0004	90.4	3.1
IFN γ	5/ 37.9	0.93	<0.0001	46.3	3.1
IL-7	#	#	0.0093	86.2	2.2
IL-12p40	1/ 20.3	1.0	<0.0001	95.8	2.1
IL-12p70	3/ 31.3	0.62	0.0187	78.9	3.2
IL-8	4/ 4.2	0.88	<0.0001	46.3	3.2
IP-10	15/ 2.3	1.0	<0.0001	0	3.9
MCP-1	15/ 3.2	1.0	<0.0001	0	0.4
MIP1 α	2/ 18.2	0.77	0.0706	72.5	3.3
MIP-1 β	9/ 19.8	0.77	0.0012	22.9	2.0
IL-4	#	##	-	96.8	3.2
IL-5	#	##	-	90.4	3.2
IL-10	5/ 8.1	0.99	<0.0001	57.2	3.1

[§] Correlations of duplicates tested using Pearson's correlation

Values for all duplicate samples were below the level of detection

Table S2. Proportion of syphilis cases presenting with symptomatic *versus* asymptomatic disease by number of syphilis infections

No. Prior Episodes Syphilis	Symptomatic (row %)	Asymptomatic	Total
0	25 (66.7)	11	36
1	22 (73.3)	8	30
2	5 (35.7)	9	14
3	1 (20)	4	5
4	2 (50)	2	4
5	0 (0)	1	1
6	0 (0)	1	1
Total	54 (59.3)	37	91

Data are n (row %)

Overall chi2(6) = 12.745, pr>chi2 = 0.0473

Chi2(1) for trend = 7.574, pr>chi2 = 0.0059

Table S3. Multivariable linear regression models for factors associated with chemo-cytokines and RPR in initial versus repeat syphilis presenters at baseline visit*

	IFN α		IL-1 β		IL-6		IL-17A		IFN γ		IL-7		IL12-p40		IL12-p70		IL-8		
	Coef.	P-value	Coef.	P-value	Coef.	P-value	Coef.	P-value	Coef.	P-value	Coef.	P-value	Coef.	P-value	Coef.	P-value	Coef.	P-value	
Syphilis Stage																			
Primary	Ref																		
Secondary	-0.11	0.845	0.05	0.412	0.00	0.996	0.34	0.094	0.10	0.748	0.05	0.827	-0.13	0.54	-0.06	0.805	0.25	0.37	
Early Latent	-0.29	0.638	0.04	0.545	0.05	0.853	0.14	0.527	-0.19	0.591	-0.09	0.725	-0.10	0.674	0.04	0.892	-0.41	0.186	
Late Latent	0.27	0.719	0.11	0.154	0.09	0.766	0.33	0.215	0.08	0.846	0.04	0.891	-0.13	0.647	0.15	0.667	-0.07	0.859	
Initial Syphilis #	0.31	0.463	-0.01	0.828	0.31	0.062	-0.01	0.959	0.10	0.688	0.32	0.063	0.18	0.265	0.11	0.558	0.34	0.103	
HIV §	0.14	0.815	-0.17	0.007	-0.20	0.392	-0.40	0.07	-0.69	0.05	0.11	0.638	0.20	0.371	-0.65	0.021	0.24	0.435	
HIV Viral Load @	0.05	0.539	0.01	0.37	0.00	0.869	0.00	0.943	0.01	0.766	0.00	0.921	0.04	0.143	0.02	0.509	-0.03	0.467	
Coinfection &	-0.55	0.56	-0.04	0.646	-0.12	0.745	-0.18	0.59	-0.40	0.462	-0.12	0.748	0.00	0.996	-0.26	0.545	0.36	0.449	
_cons	1.33	0.063	0.56	0	0.48	0.085	0.71	0.006	1.76	0	0.20	0.482	-0.12	0.657	1.23	0	1.02	0.005	

	IP-10		MCP1		MIP1 α		MIP1 β		IL-4		IL-5		IL-10		CRP		RPR	
	Coef.	P-value	Coef.	P-value	Coef.	P-value	Coef.	P-value	Coef.	P-value	Coef.	P-value	Coef.	P-value	Coef.	P-value	Coef.	P-value
Syphilis Stage																		
Primary																		
Secondary	-0.02	0.905	-0.12	0.282	0.22	0.434	-0.01	0.97	0.21	0.201	-0.02	0.948	-0.56	0.175	-0.12	0.843	0.31	0.043
Early Latent	-0.22	0.303	-0.11	0.352	-0.35	0.264	-0.84	0.046	0.10	0.587	-0.26	0.318	-1.67	0.001	-1.36	0.052	-0.03	0.866
Late Latent	-0.30	0.254	-0.29	0.049	0.02	0.952	-0.15	0.759	0.14	0.515	0.13	0.671	-1.66	0.003	-1.83	0.032	0.09	0.647
Initial Syphilis #	0.19	0.197	0.03	0.696	0.36	0.089	0.54	0.057	-0.08	0.537	0.26	0.147	1.29	0.001	0.29	0.536	-0.44	0.001
HIV §	0.39	0.067	0.01	0.909	0.37	0.227	1.07	0.01	-0.43	0.016	0.28	0.27	1.70	0.001	0.61	0.369	-0.08	0.643
HIV Viral Load @	0.05	0.043	0.02	0.142	-0.06	0.086	-0.12	0.021	0.02	0.265	-0.03	0.374	-0.03	0.603	0.10	0.264	0.03	0.174
Coinfection &	0.26	0.428	-0.01	0.973	0.12	0.804	-1.22	0.056	-0.15	0.588	0.05	0.895	0.99	0.151	0.40	0.701	0.16	0.523
_cons	6.21	0	5.53	0	0.85	0.019	2.23	0	0.75	0.001	0.46	0.124	0.95	0.07	2.85	0	2.54	0

Values of all chemo- and cytokines have been log transformed, CRP and RPR were square root transformed. Raw coefficients are provided for the explanatory variables.

Comparison group is those with Repeat syphilis at baseline visit.

§ HIV coded as 0 = negative, 1 = positive

@ HIV Viral load in copies per mL. Continuous variable- log transformed

& Presence of *N. gonorrhoeae* or *C. trachomatis* at baseline visit. Coded 0 = both absent, 1 = either present.

Table S4. Baseline chemo-, cytokine and RPR values in 3 individuals with a positive serum *T. pallidum* PCR but latent syphilis.

Participant	Primo*	IFN α	IFN γ	IL1 β	IL12p40	IL12p70	IP10	MCP1	MIP1 α	MIP1 β	IL4	IL5	IL6	IL7	IL8	IL10	IL17A	CRP	RPR	RPR Delta
1	Init.	0.8	1.545	1.57	1.025	1.61	443.98	306.05	1.63	12.08	1.575	1.585	1.26	1.37	1.605	3.27	1.525	5.2	1	
2	Reinfec.	16.43	1.545	1.57	1.025	1.61	3979.6	406.98	4.64	23.05	1.575	1.585	1.26	1.11	4.3	64.81	1.525	15.2	1/64	
3	Init.	0.8	1.545	1.57	1.025	1.61	885.12	265.31	1.63	8.11	1.575	1.585	1.26	1.11	1.605	14.2	1.525	18.4	1/32	

* Init.- Initial infection, Reinfec. – reinfection.

Delta RPR – Change in RPR titre between baseline and 6-month visit

Figure S1. Flow chart showing participant recruitment

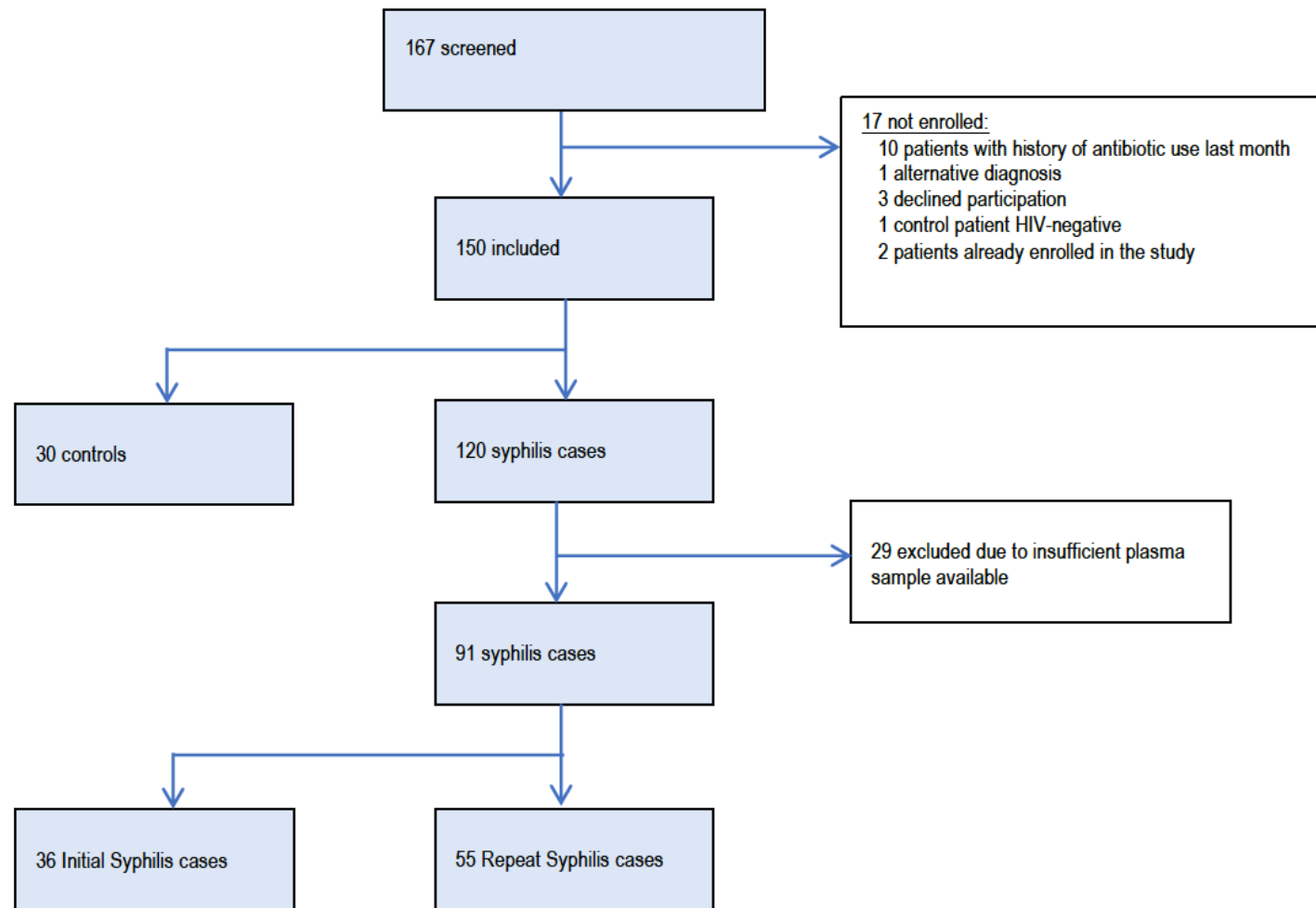


Figure S2. RPR titre by number of episodes of syphilis in retrospective study. RPR levels are shown as median and interquartile range (box), with 10_{th} and 90_{th} percentiles (whiskers).

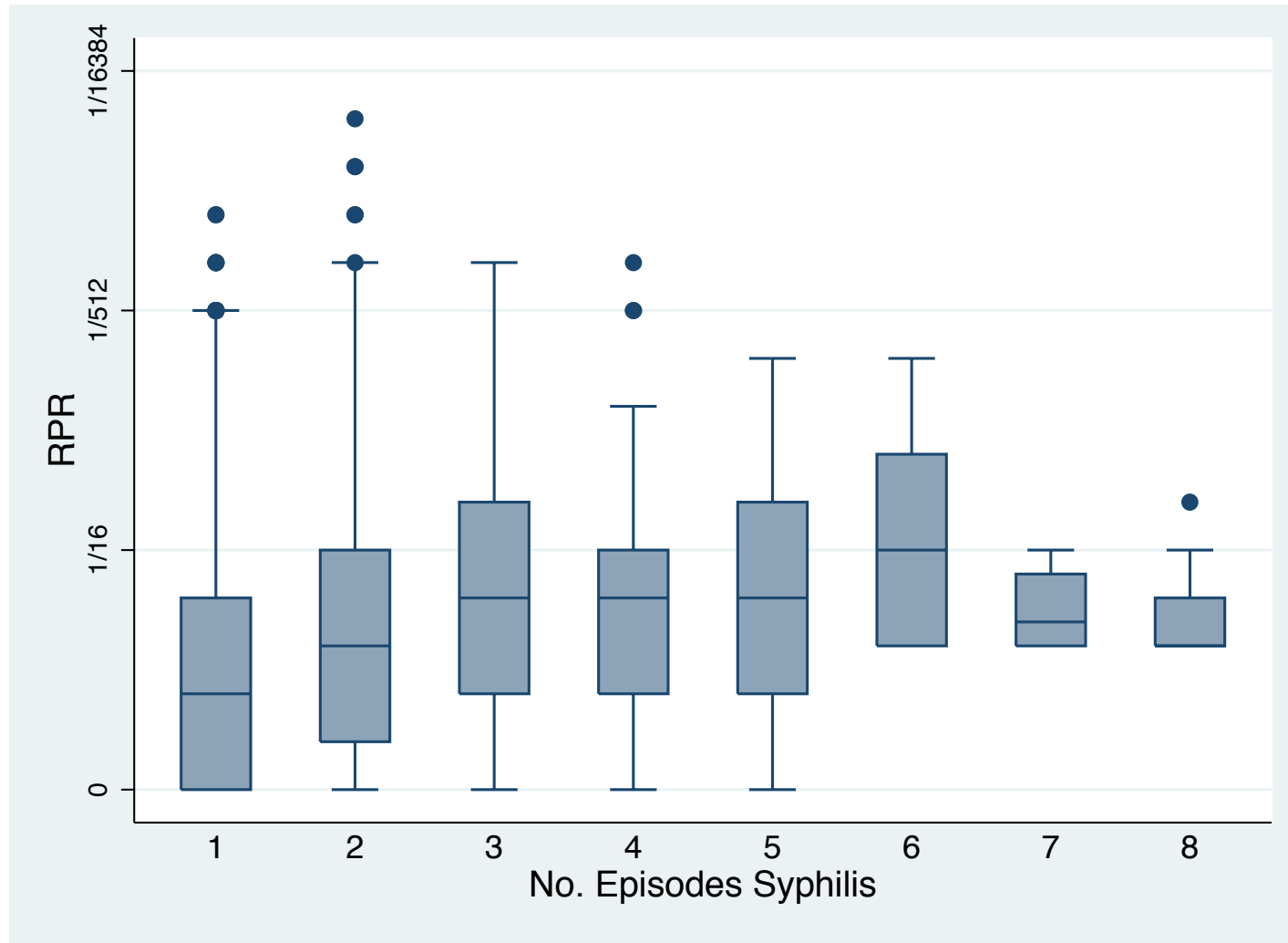


Figure S3. Rapid Plasma Reagin (RPR) titre profiles by time of testing for 20 individuals with four or more episodes of syphilis during course of retrospective study follow-up.

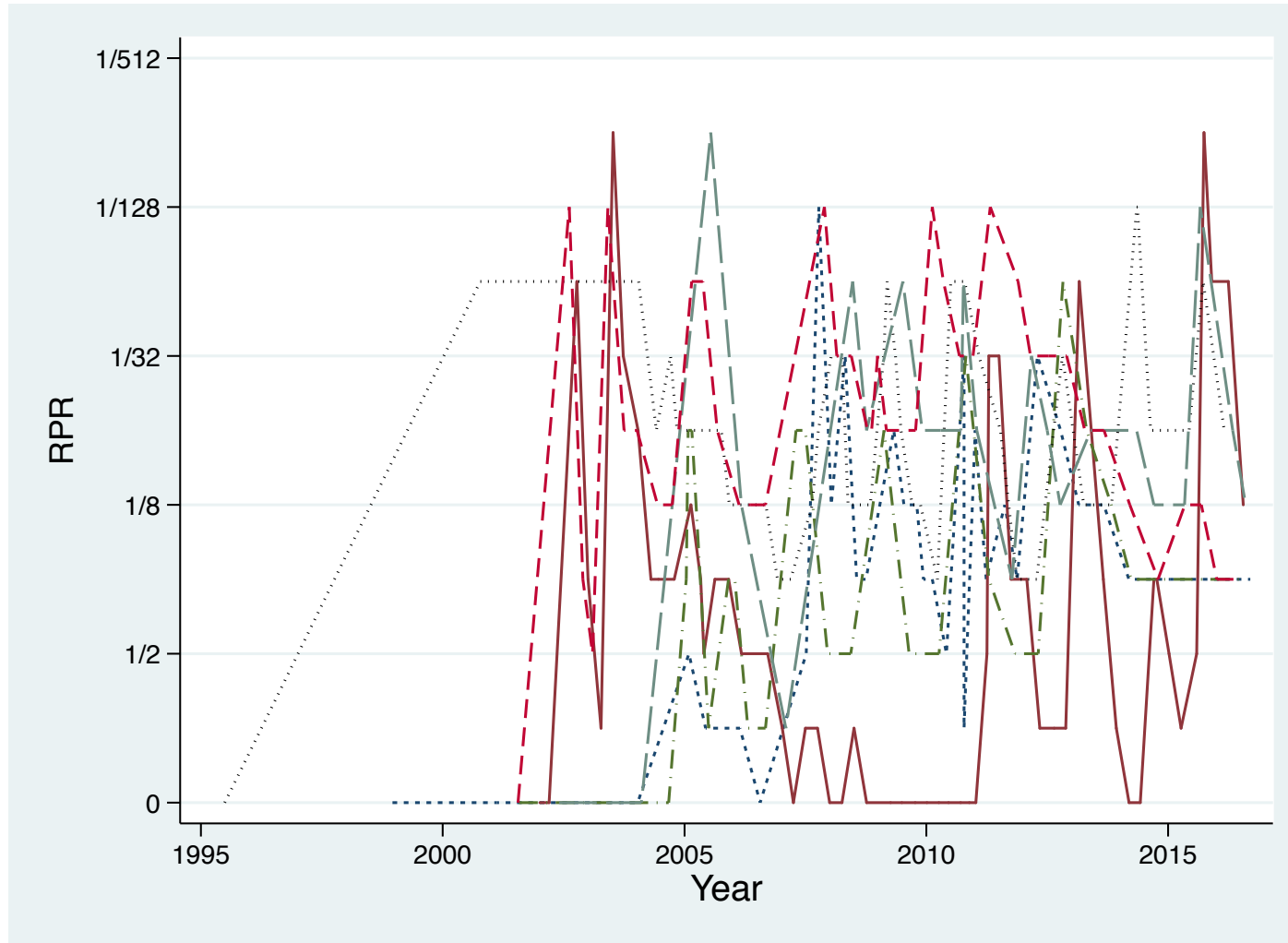
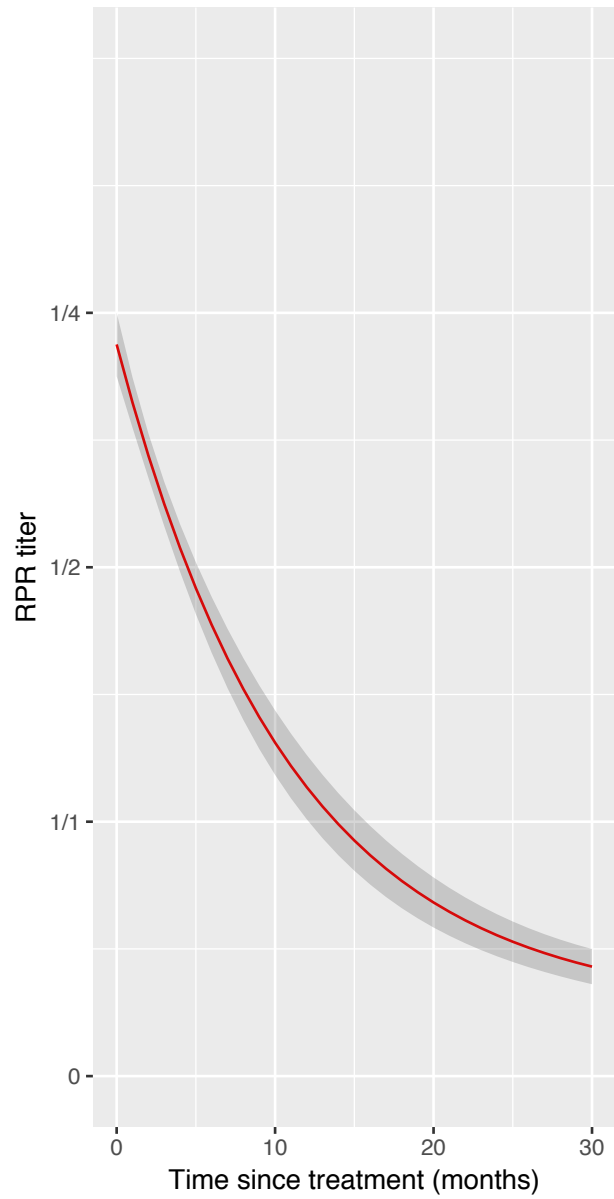
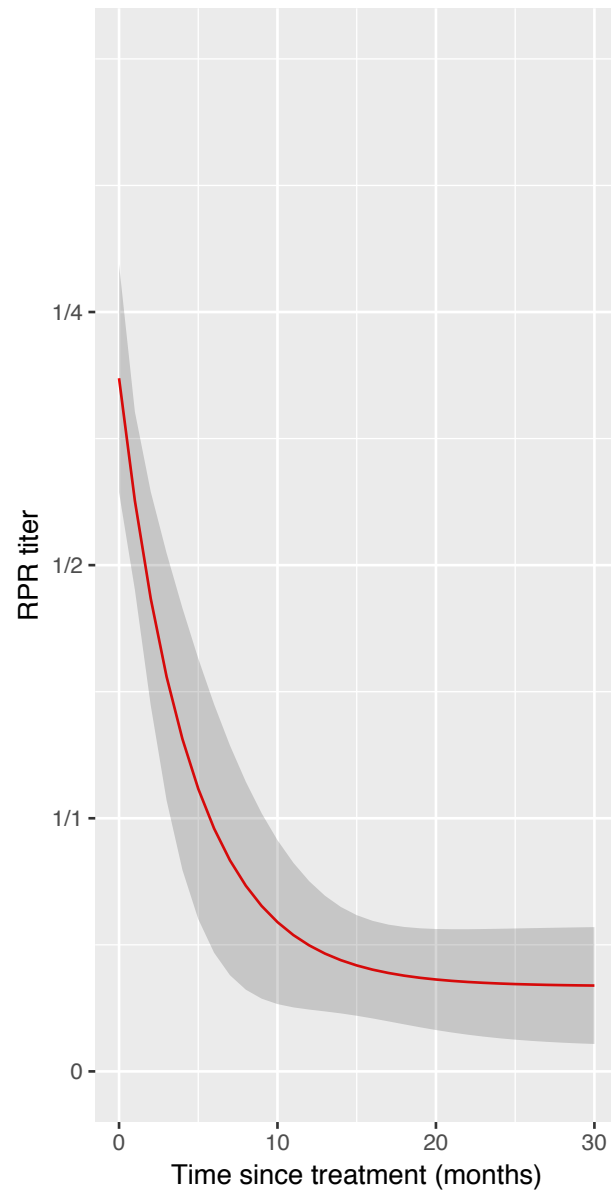


Figure S4a. Curves of Rapid Plasma Reagin (RPR) decline in initial versus repeat syphilis in individuals attending HIV/STI clinic 1993-2016 (retrospective series), *utilizing the same initial RPR values at initial and repeat infections*. Each pair of graphs is for the group of individuals with a particular RPR value at initial or repeat infection from 1/2 to 1/1024. Thus the 'Initial RPR 1/8' graph depicts in the 'Initial infections' subgraph the RPR curve for the syphilis episodes that had an RPR of 1/8 at initial syphilis diagnosis. In the 'Repeat infections' subgraph it depicts the RPR curve for all episodes of repeat syphilis where the RPR was 1/8 at the time of the reinfections.

Initial infections

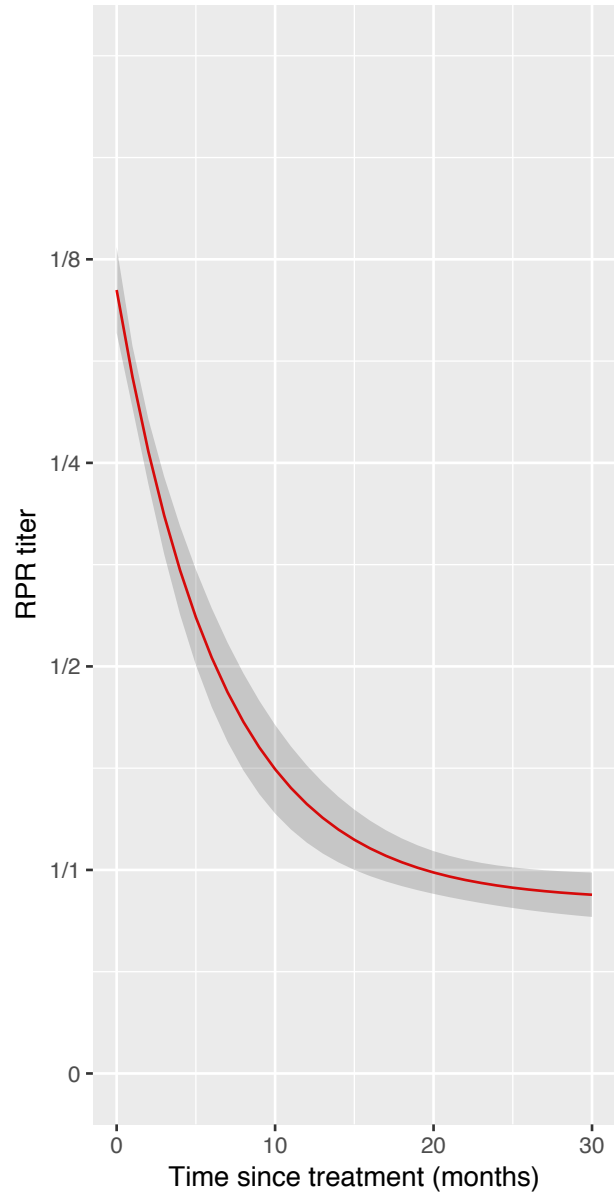


Repeat infections

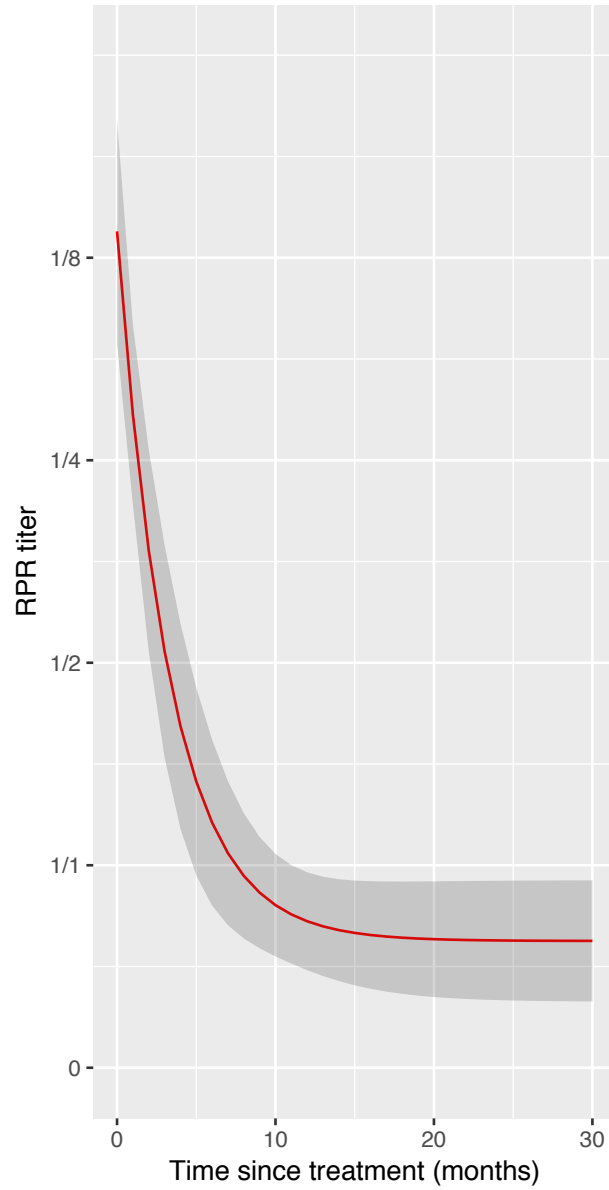


Initial RPR 1/4

Initial infections

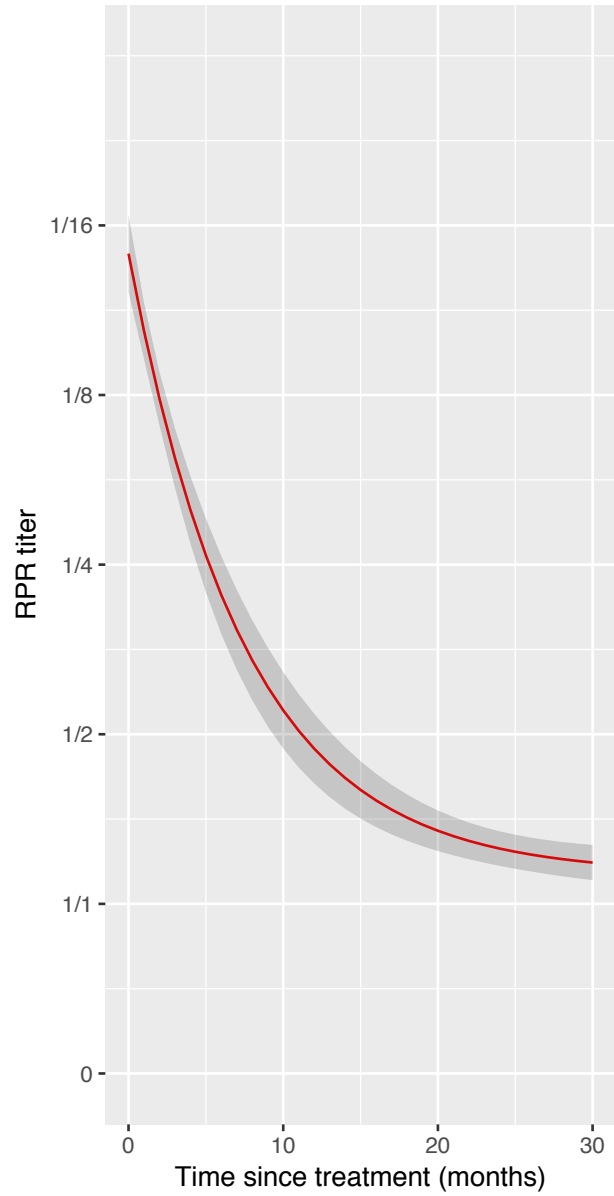


Repeat infections

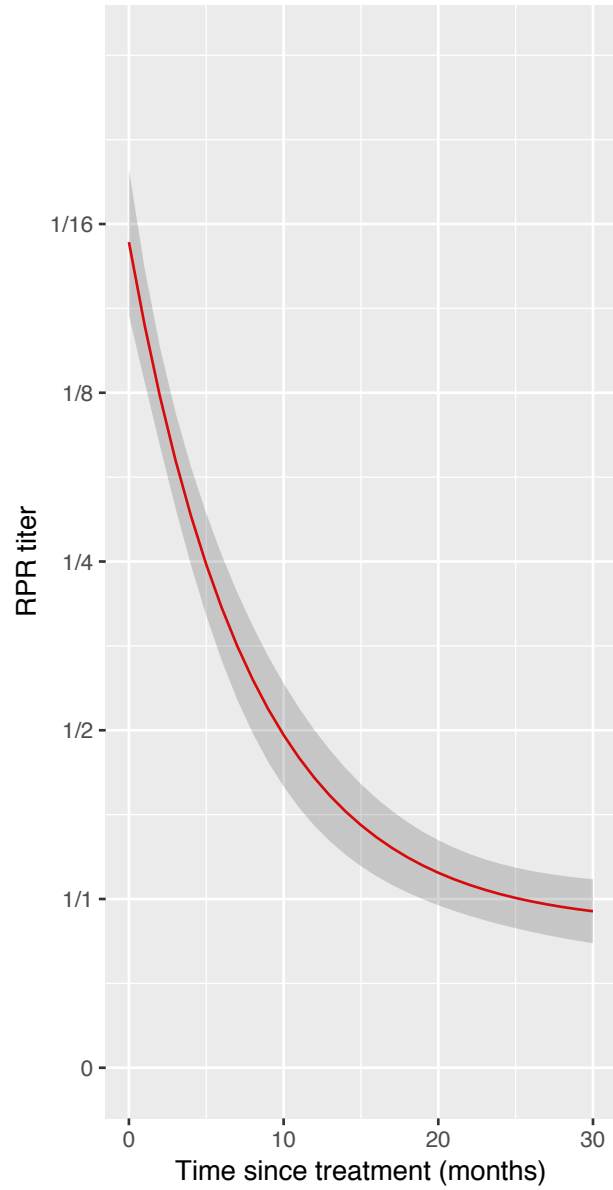


Initial RPR 1/8

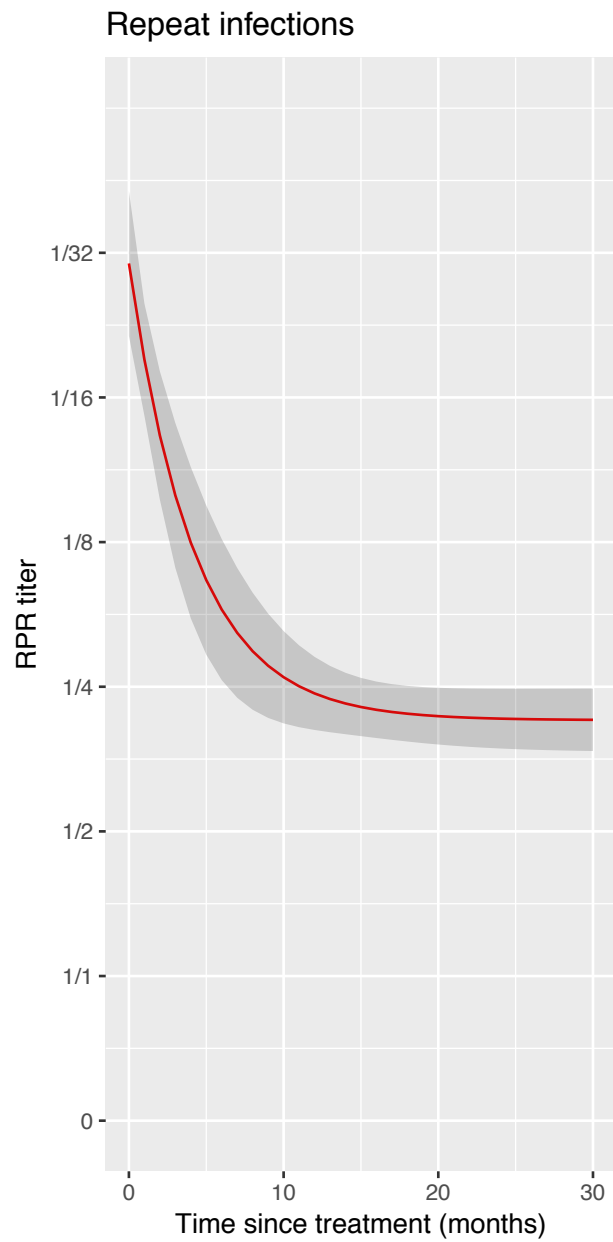
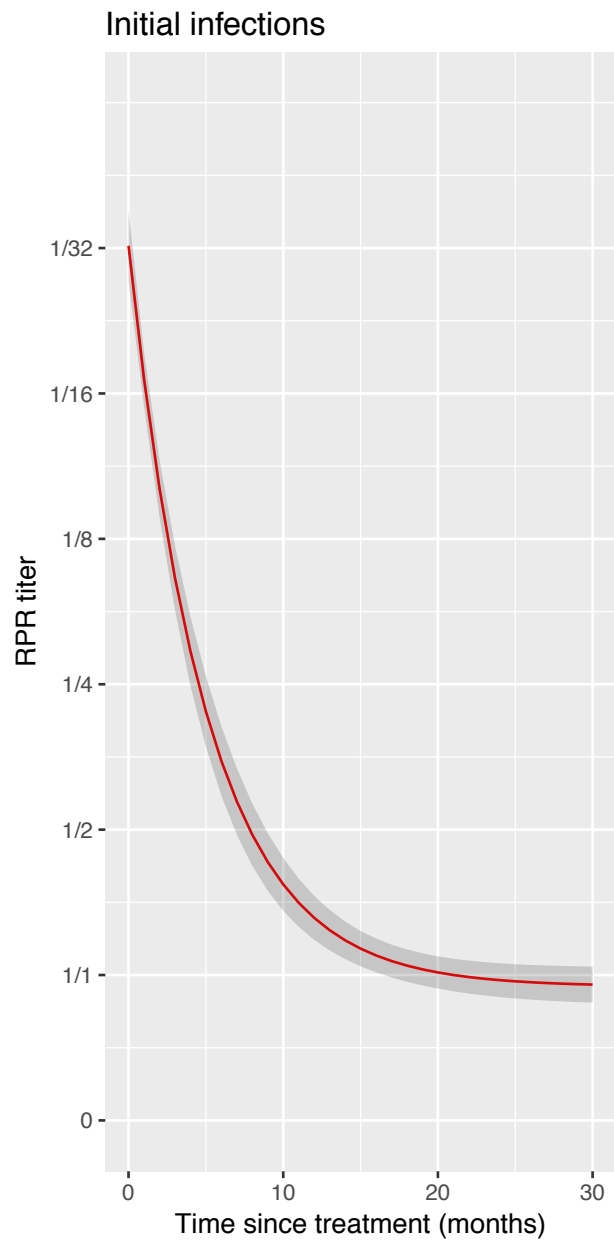
Initial infections



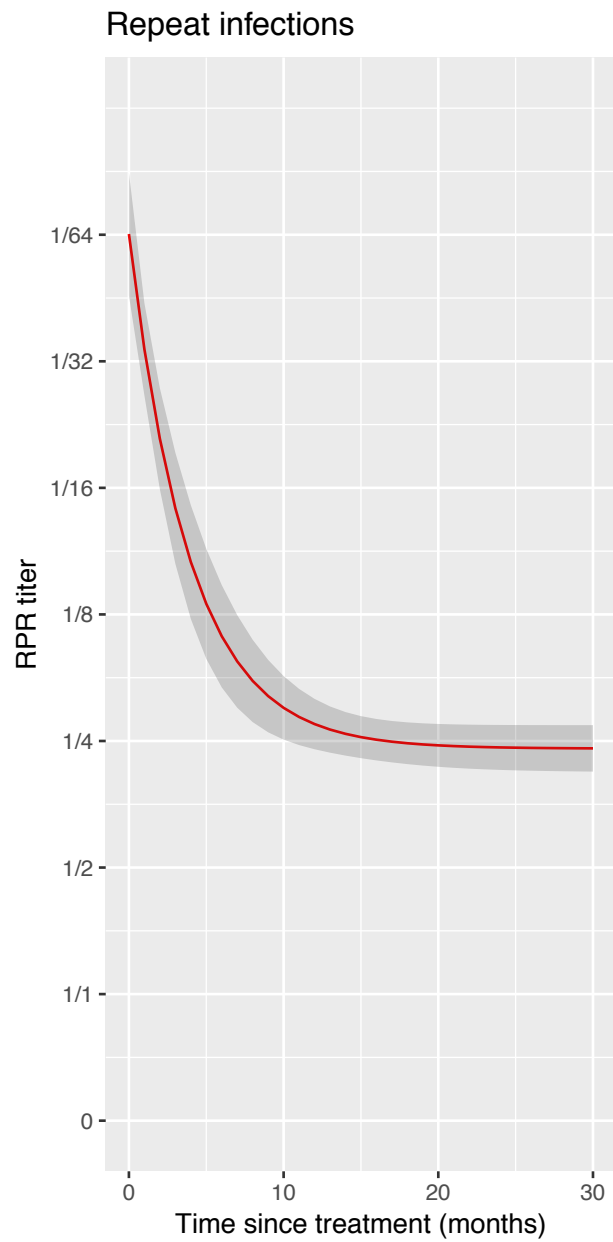
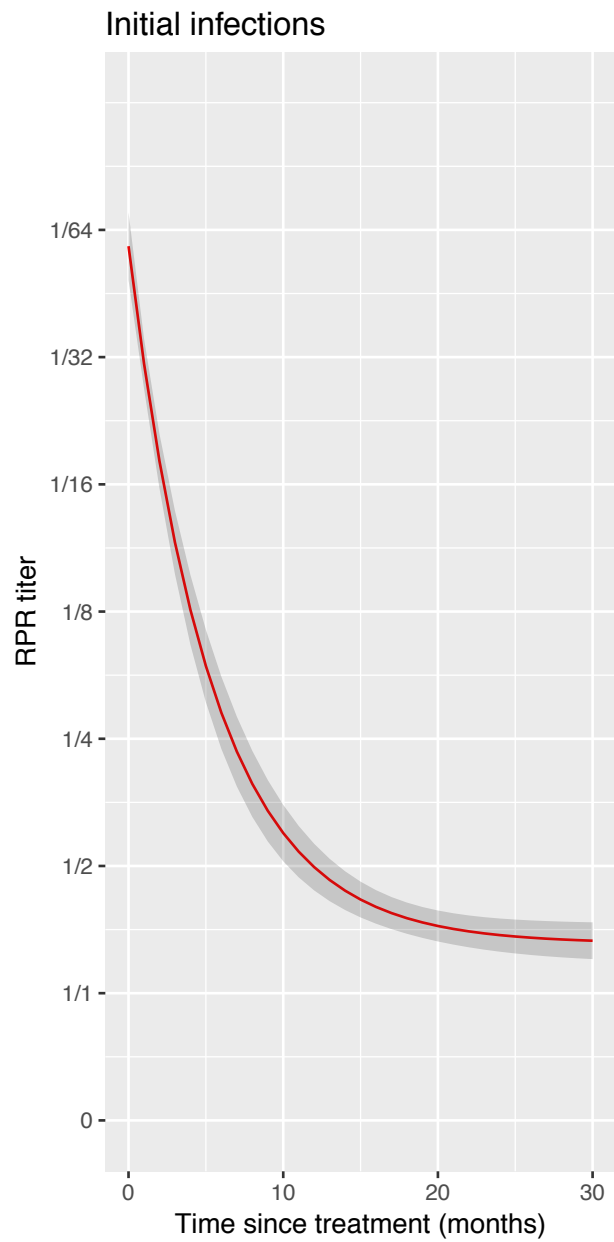
Repeat infections



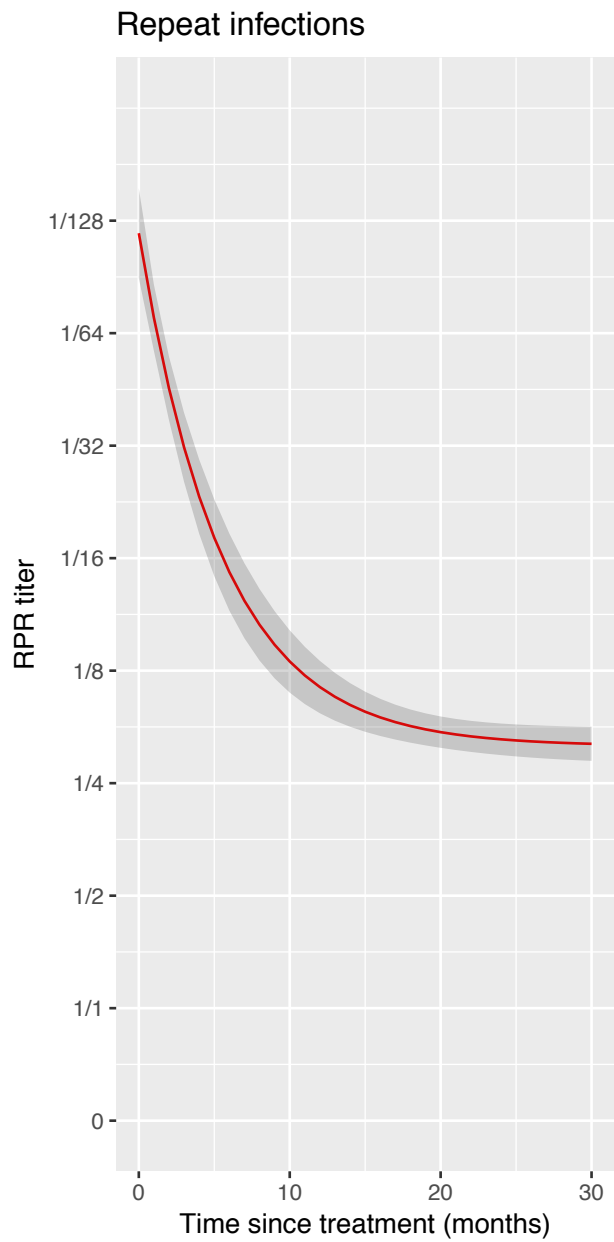
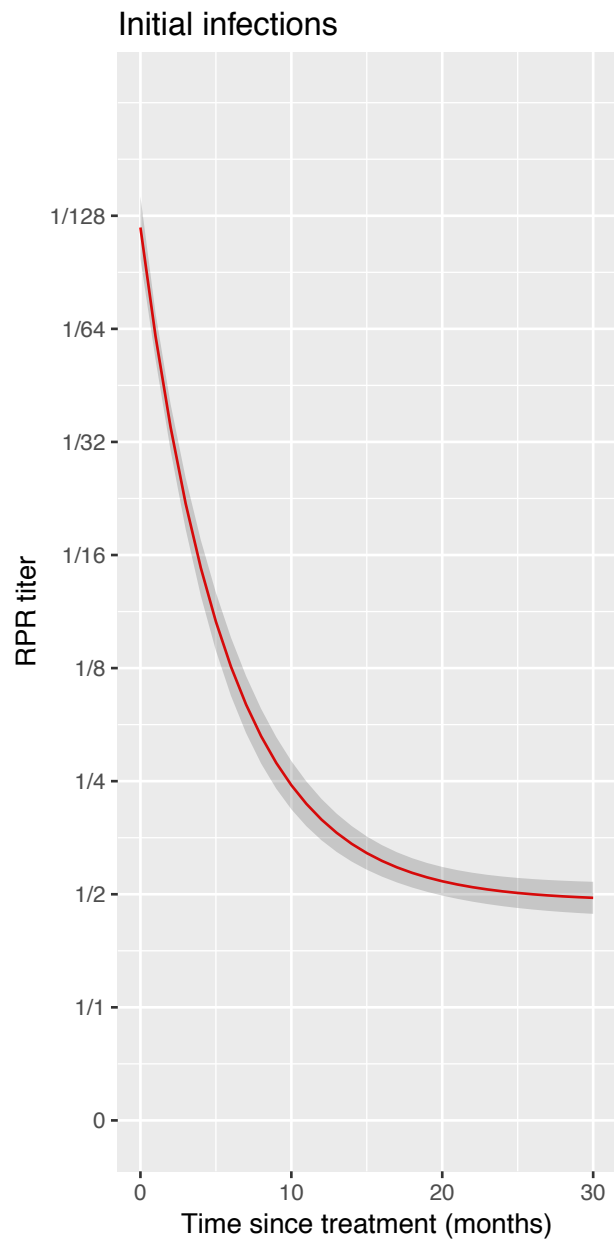
Initial RPR 1/16



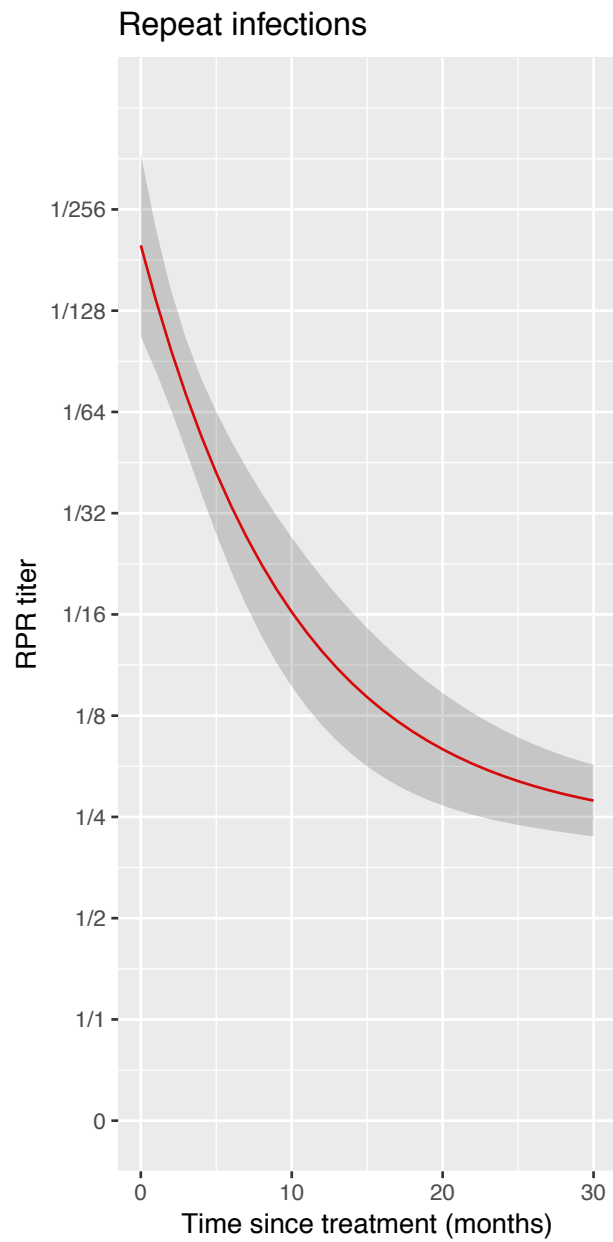
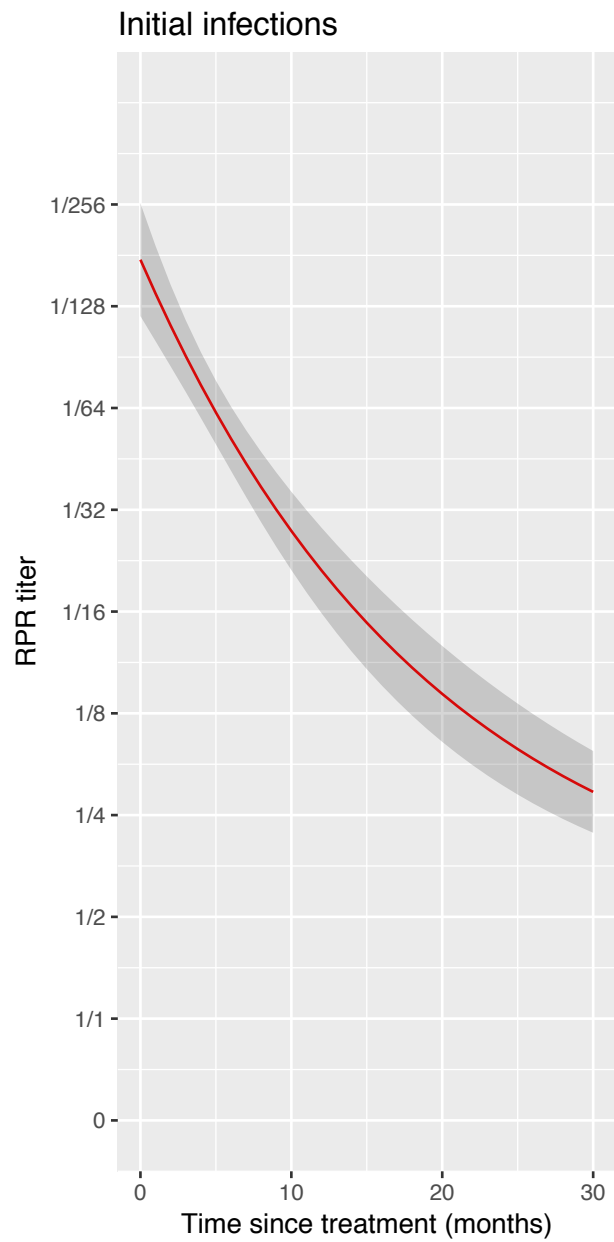
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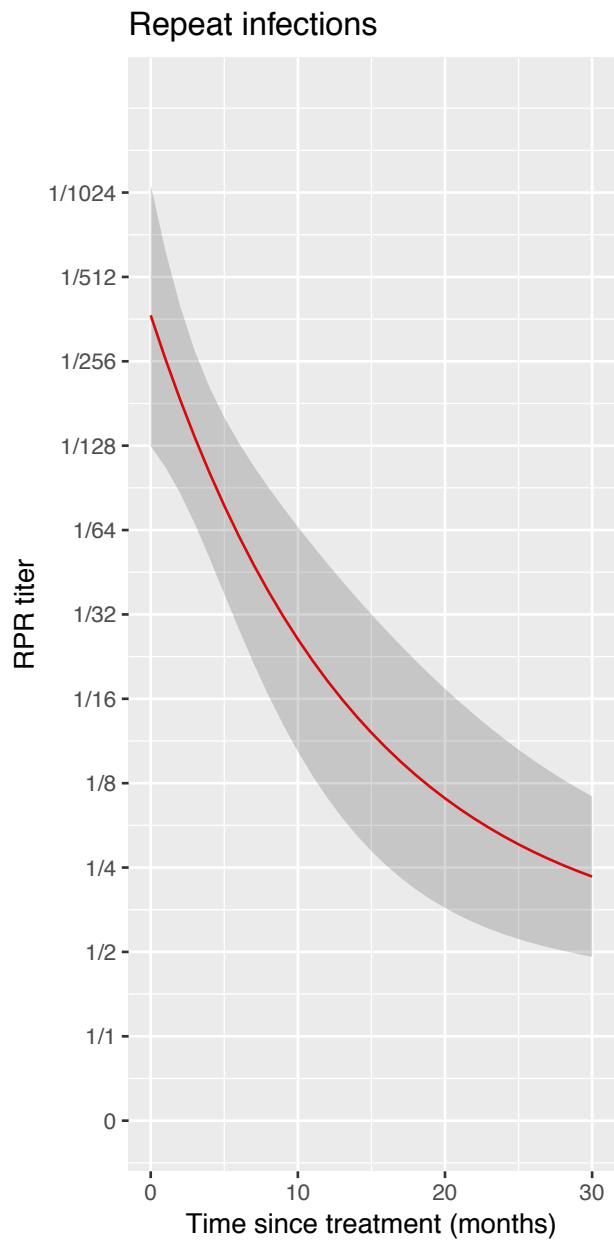
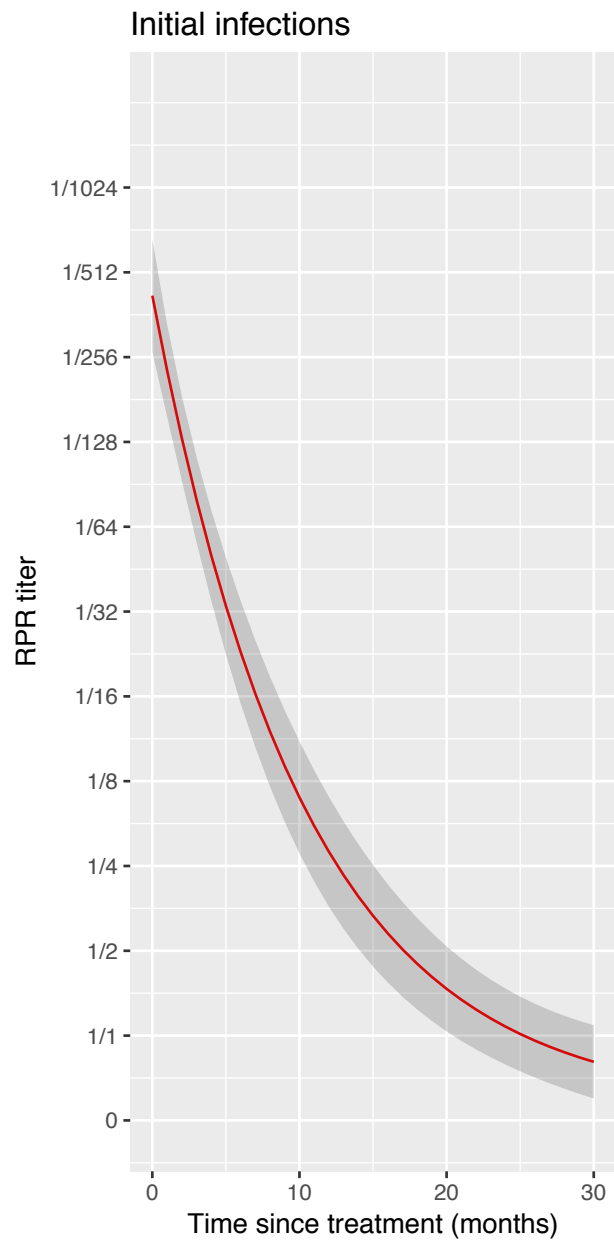
Initial RPR 1/64



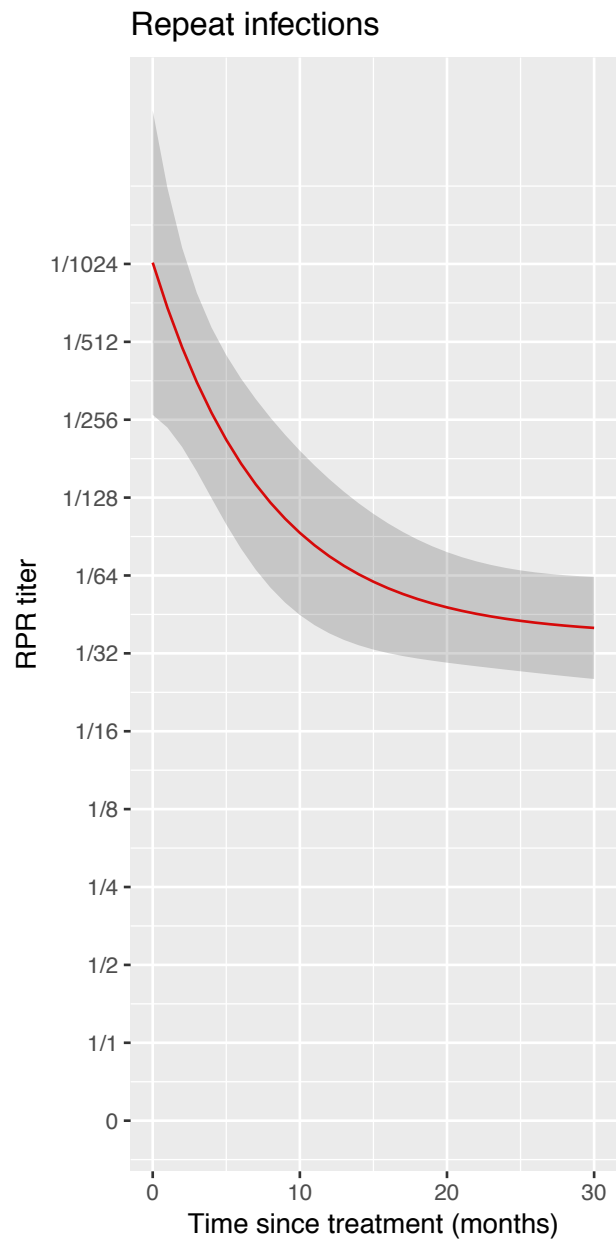
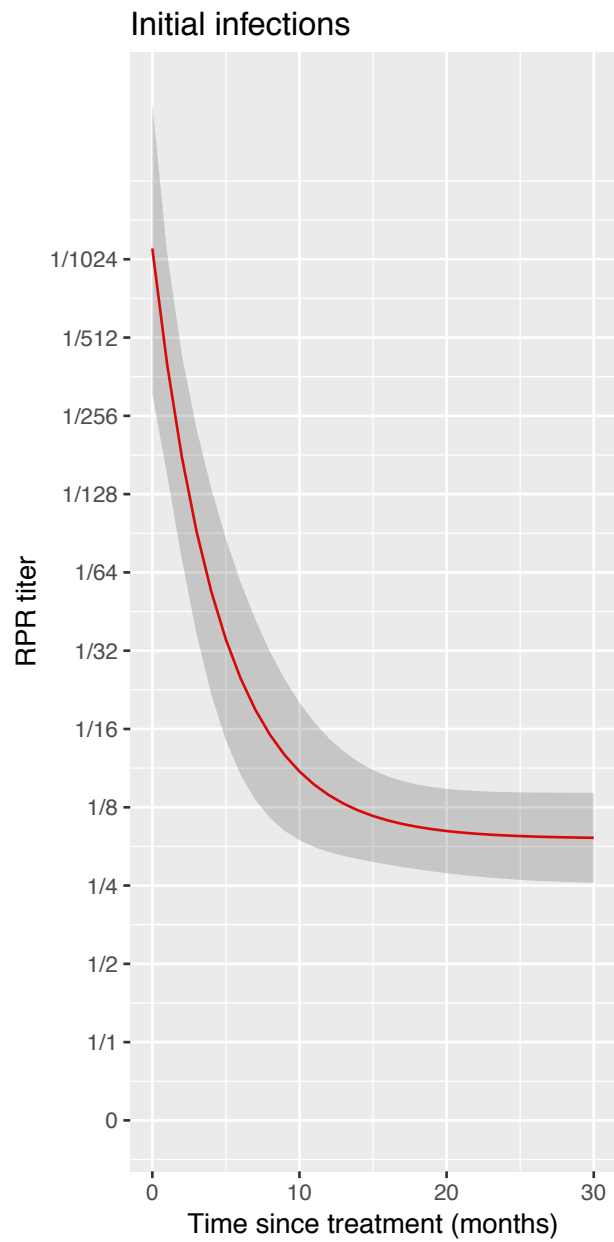
Initial RPR 1/128



Initial RPR 1/256

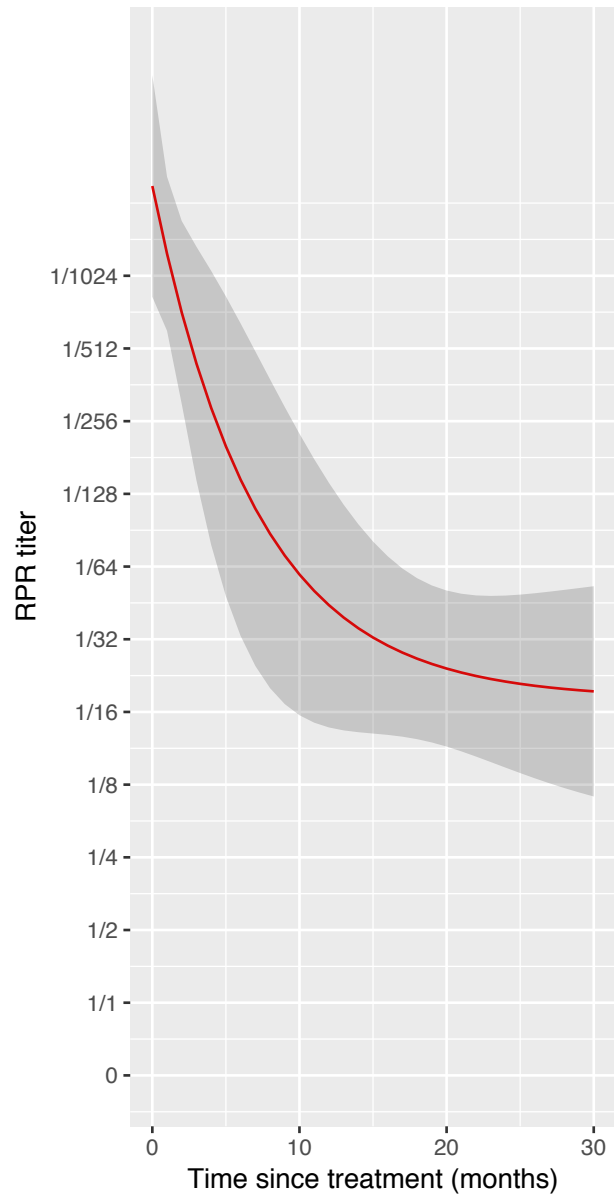


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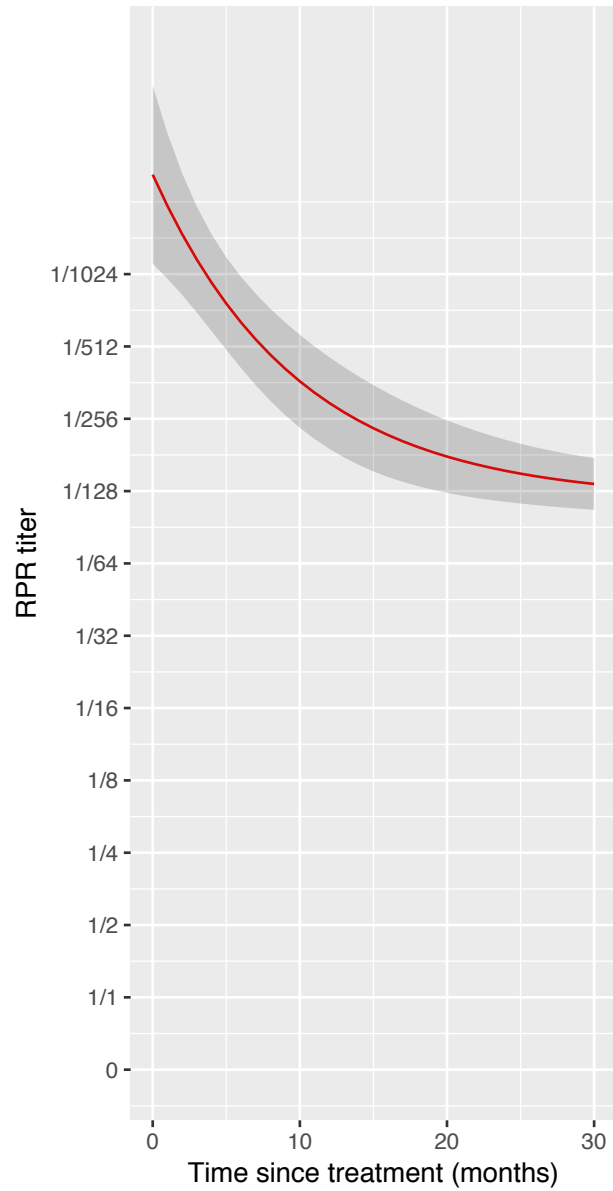


Initial RPR 1/1024

Initial infections

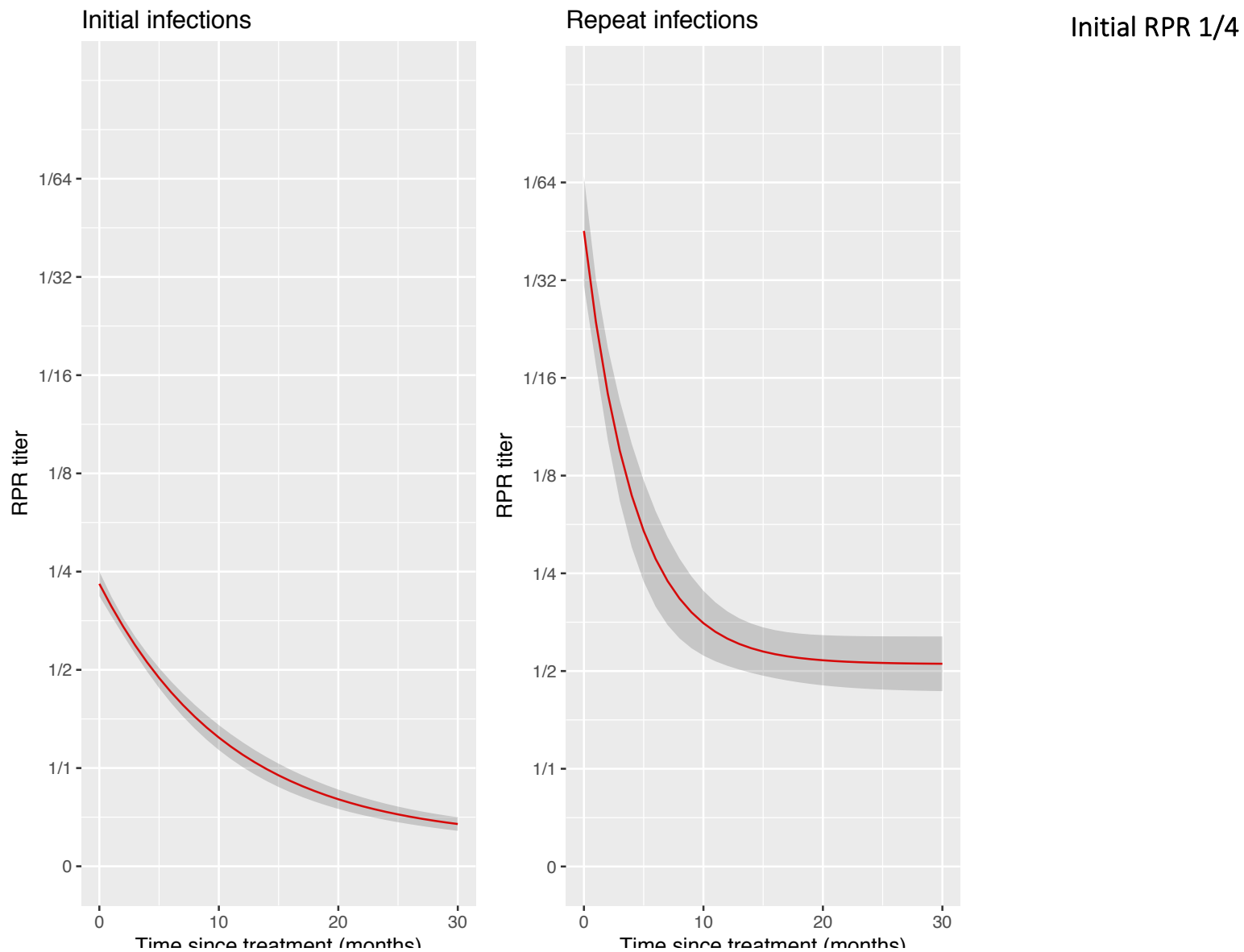


Repeat infections

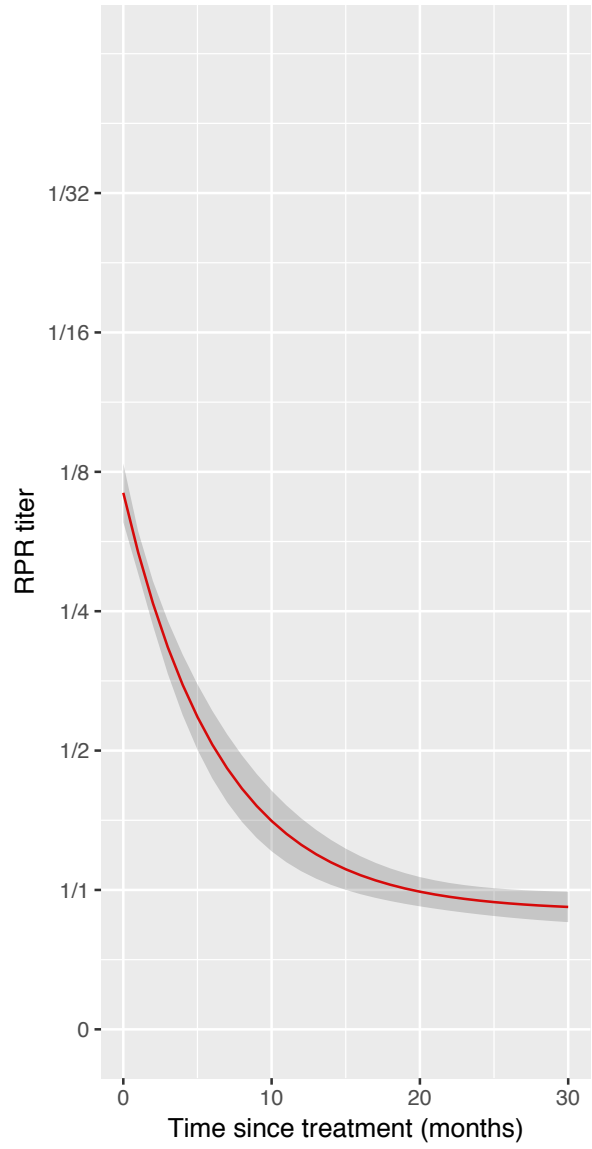


Initial RPR 1/2048

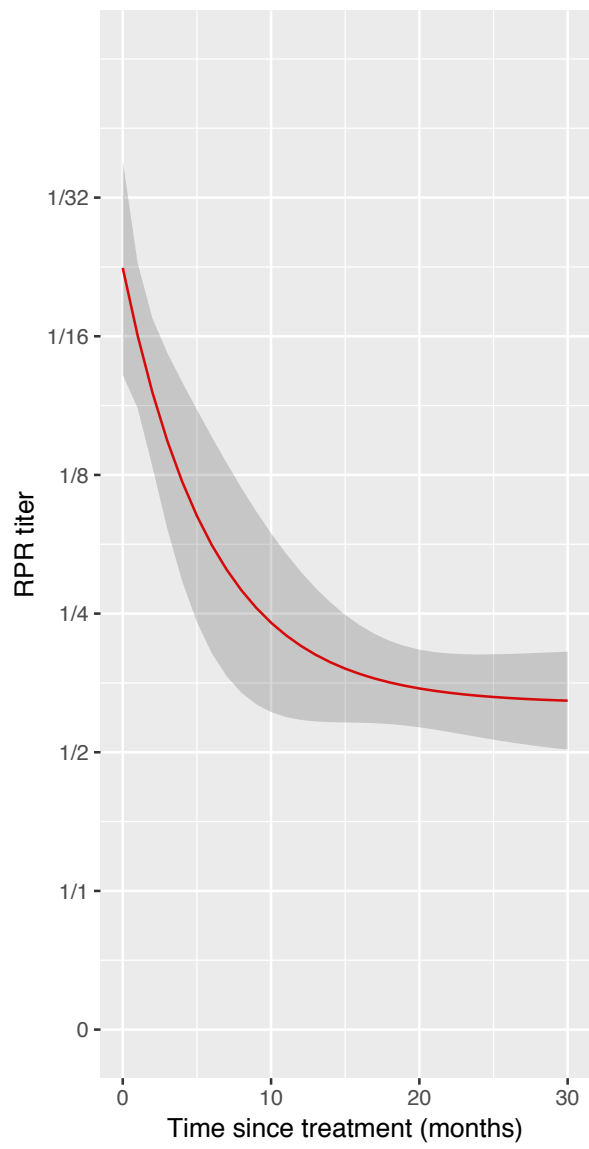
Figure S4b. Curves of Rapid Plasma Reagin (RPR) decline in initial versus repeat syphilis in individuals attending HIV/STI clinic 1993-2016 (retrospective series), *utilizing the same individuals at initial and repeat infections*. Each pair of graphs is for the group of individuals with a particular initial RPR value from 1/2 to 1/1024. Thus the 'Initial RPR 1/2' graph depicts in the 'Initial infections' subgraph the RPR curve for the syphilis episodes that had an RPR of 1/2 at initial syphilis diagnosis. In the 'Repeat infections' subgraph it depicts the RPR curve that this same group of persons had at the time of their reinfections.



Initial infections

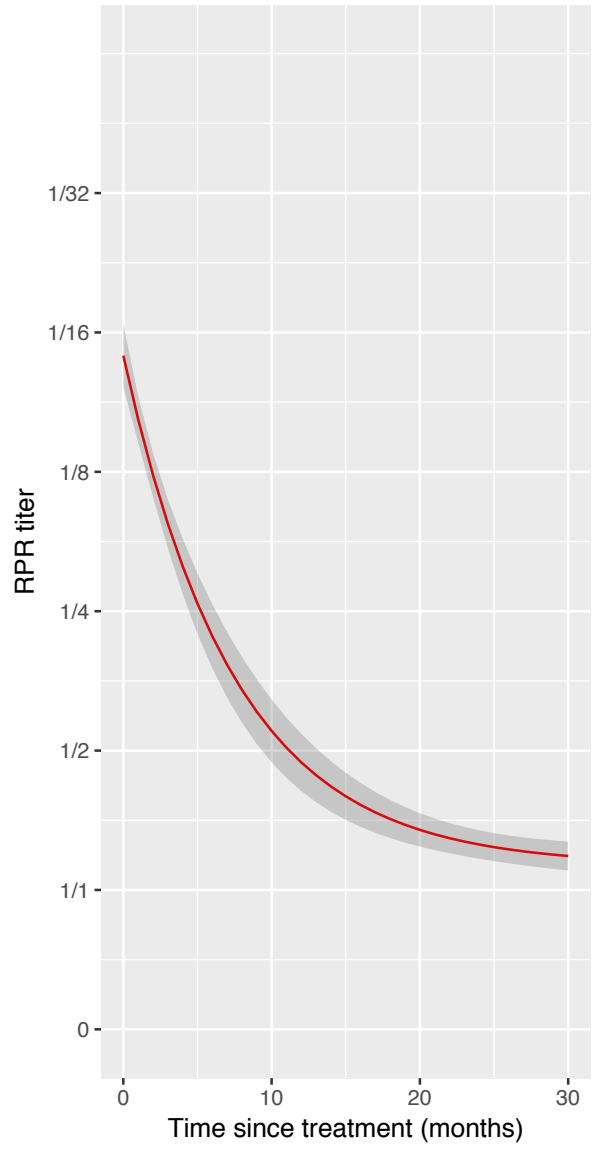


Repeat infections

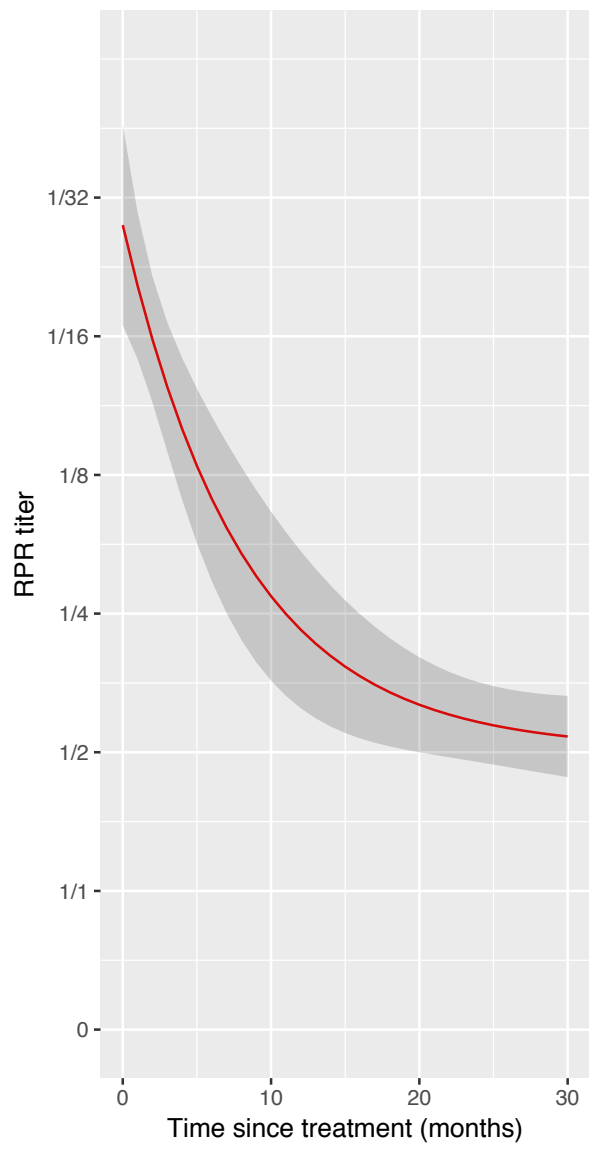


Initial RPR 1/8

Initial infections

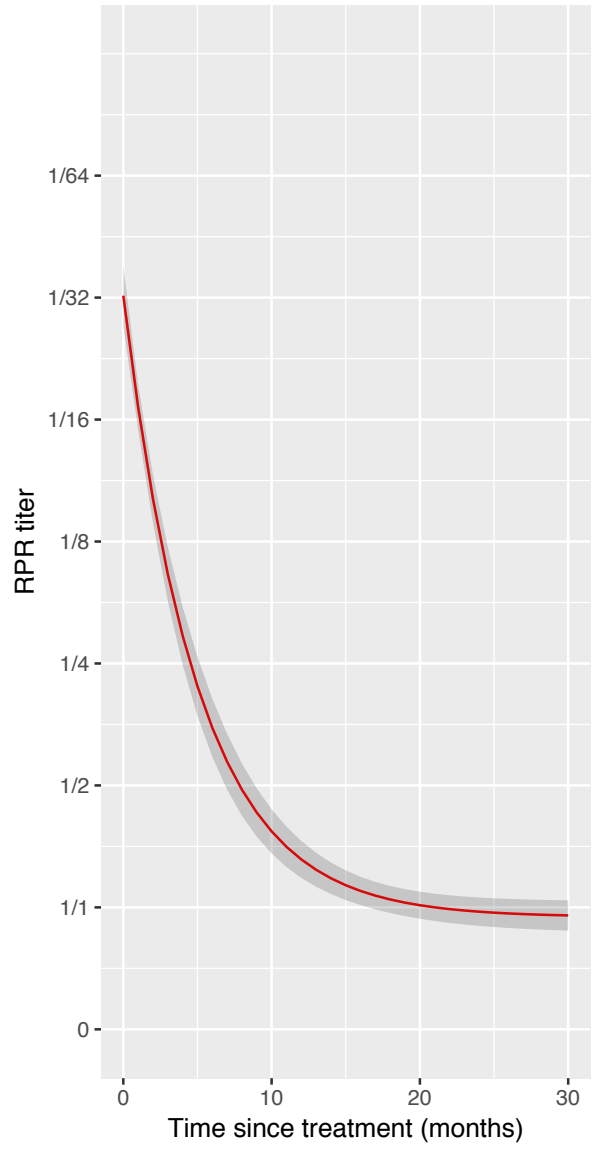


Repeat infections

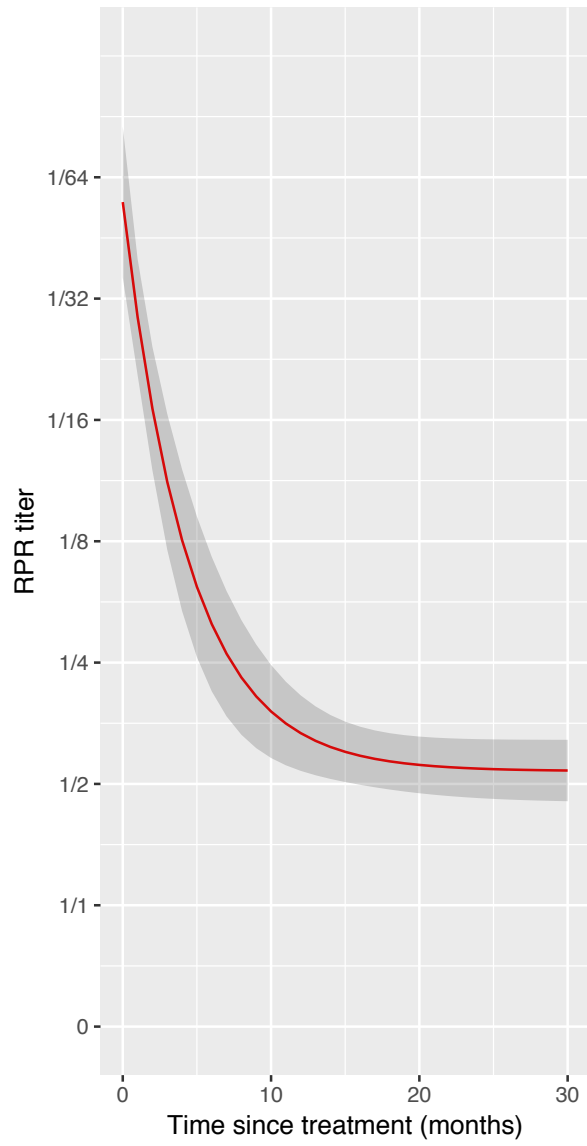


Initial RPR 1/16

Initial infections

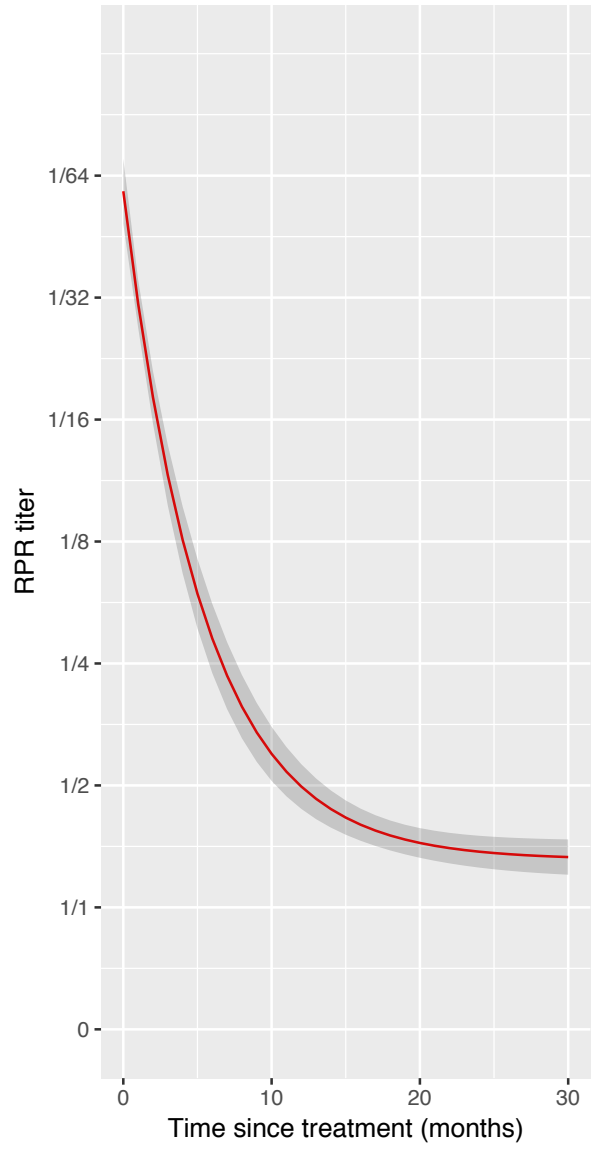


Repeat infections

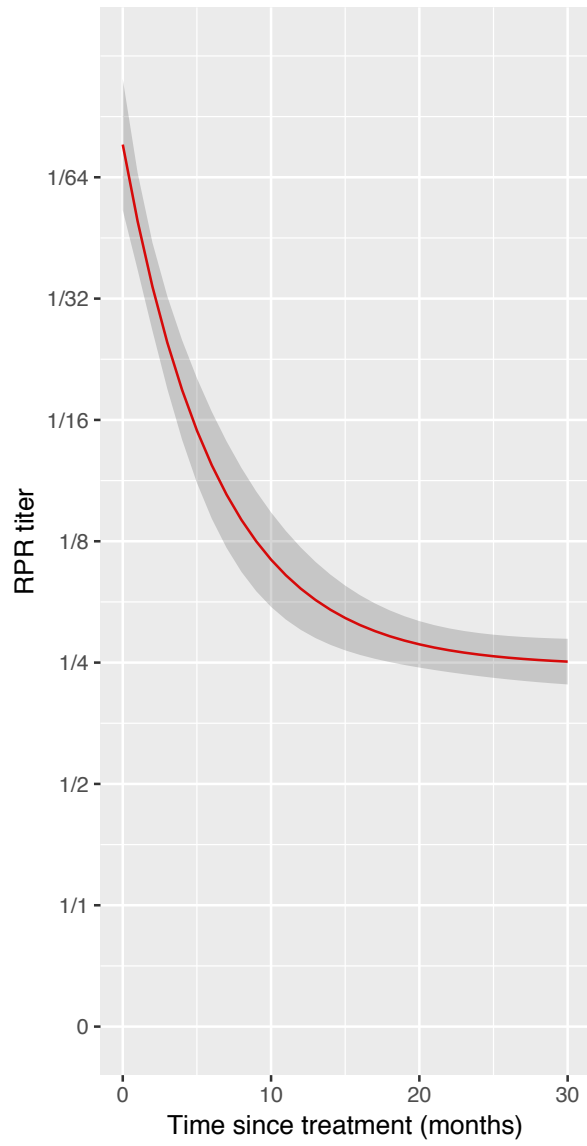


Initial RPR 1/32

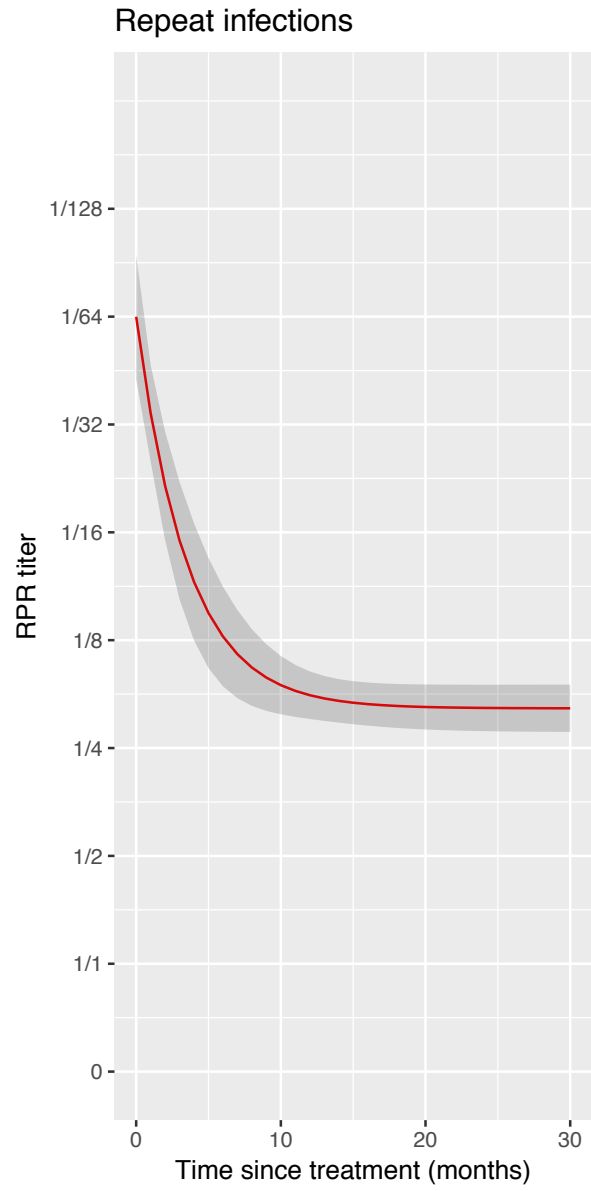
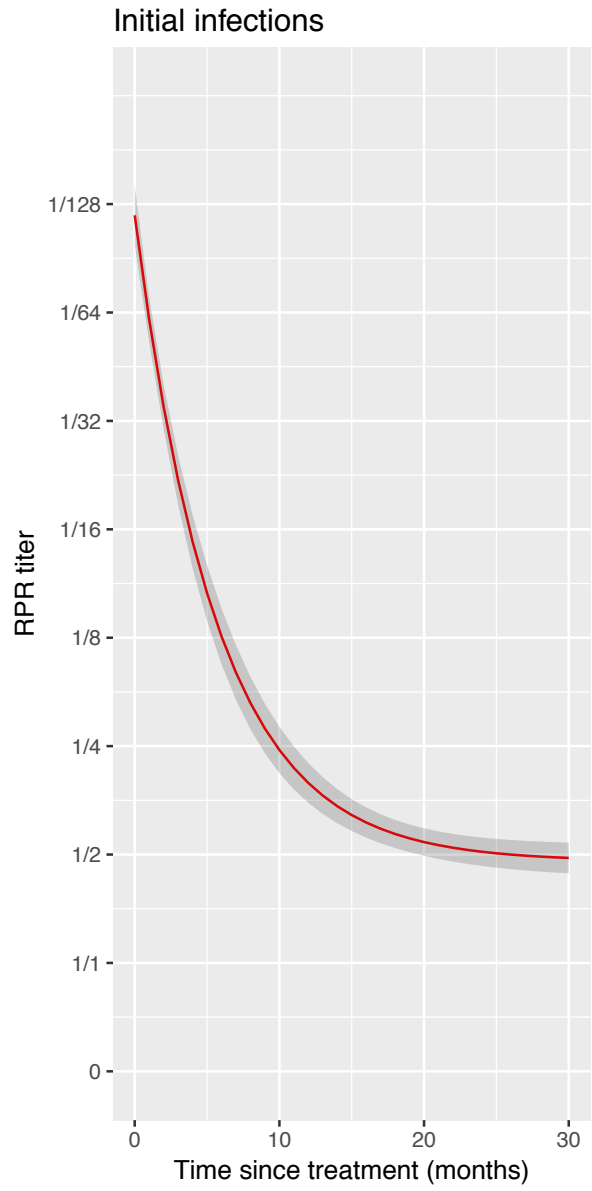
Initial infections



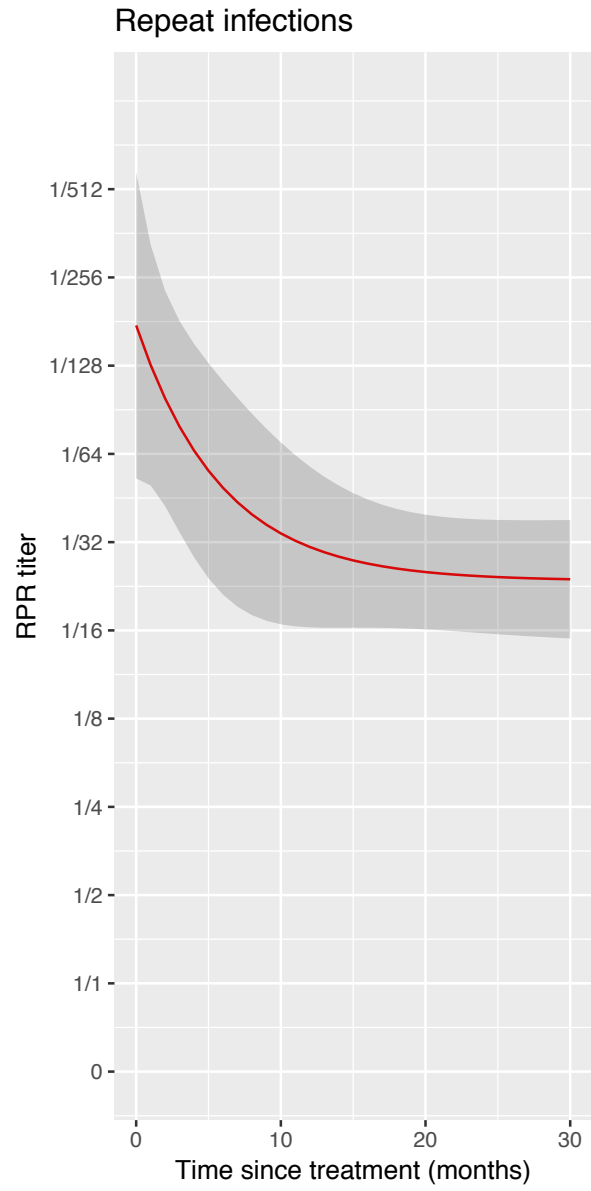
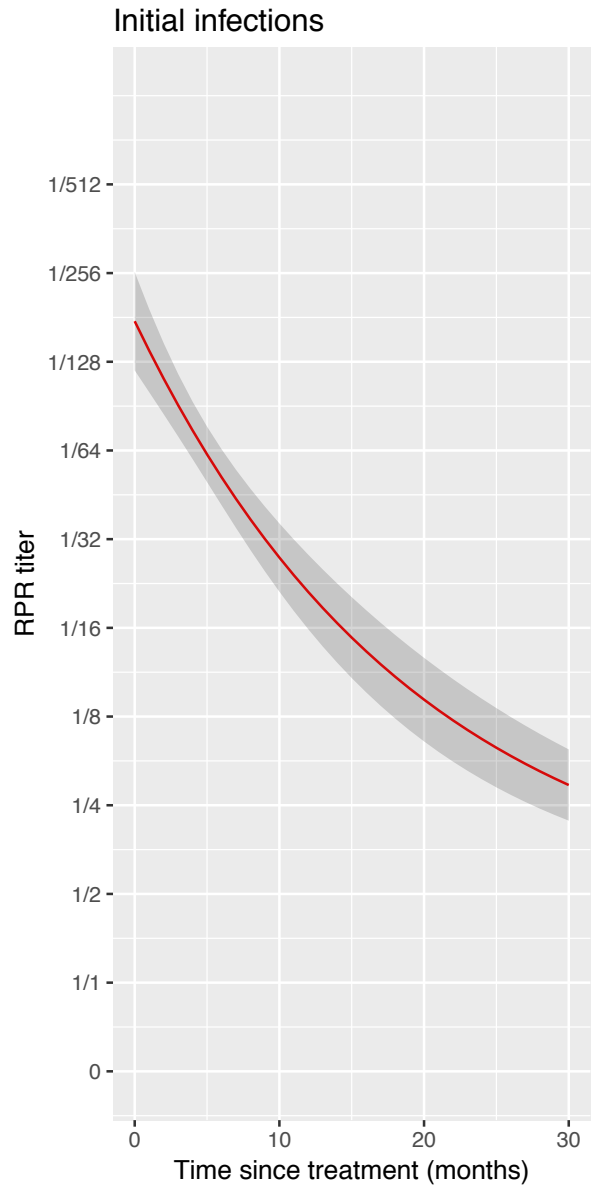
Repeat infections



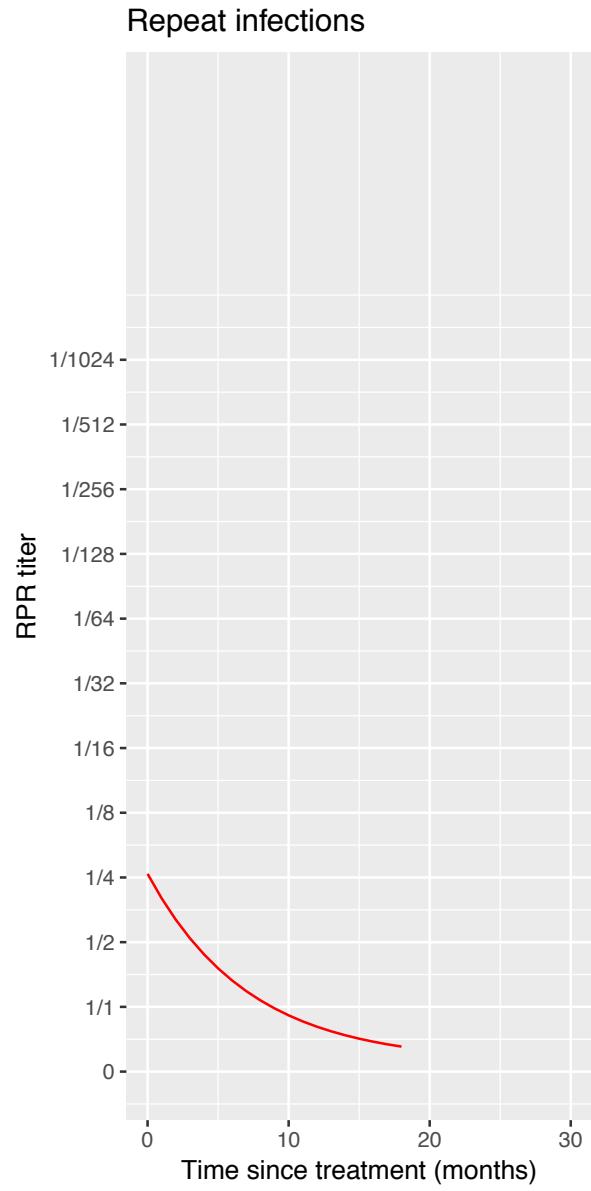
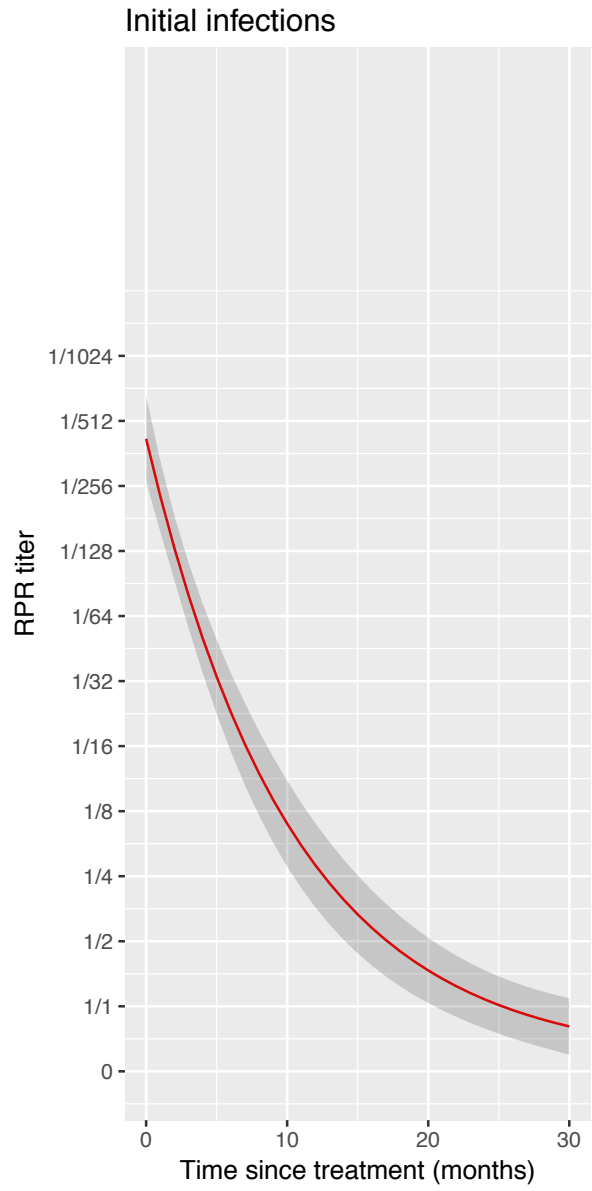
Initial RPR 1/64



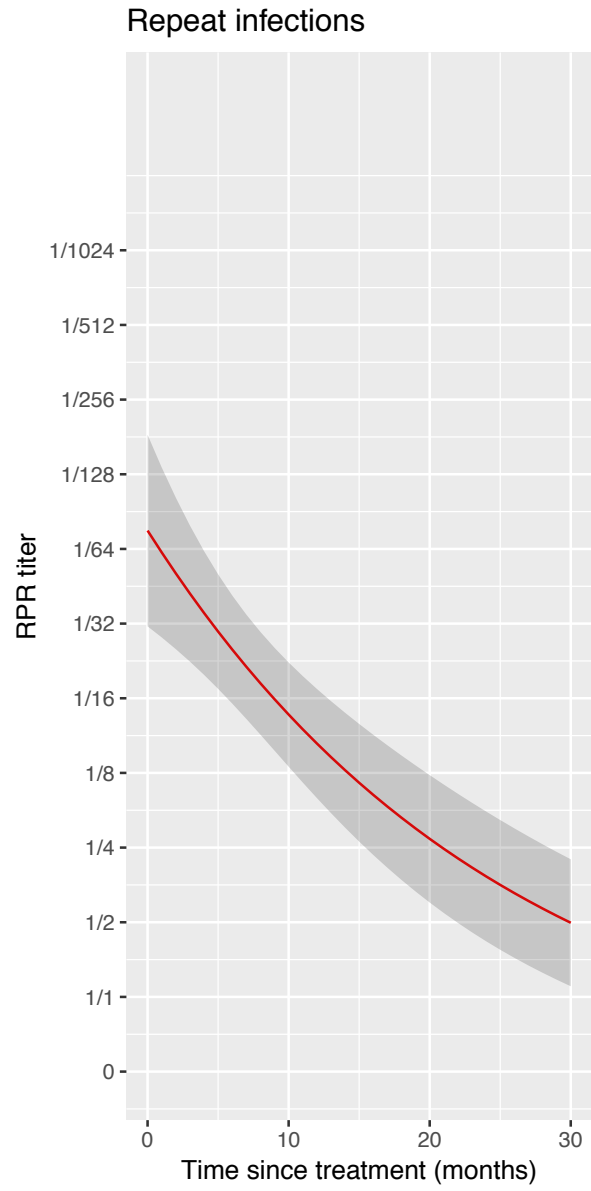
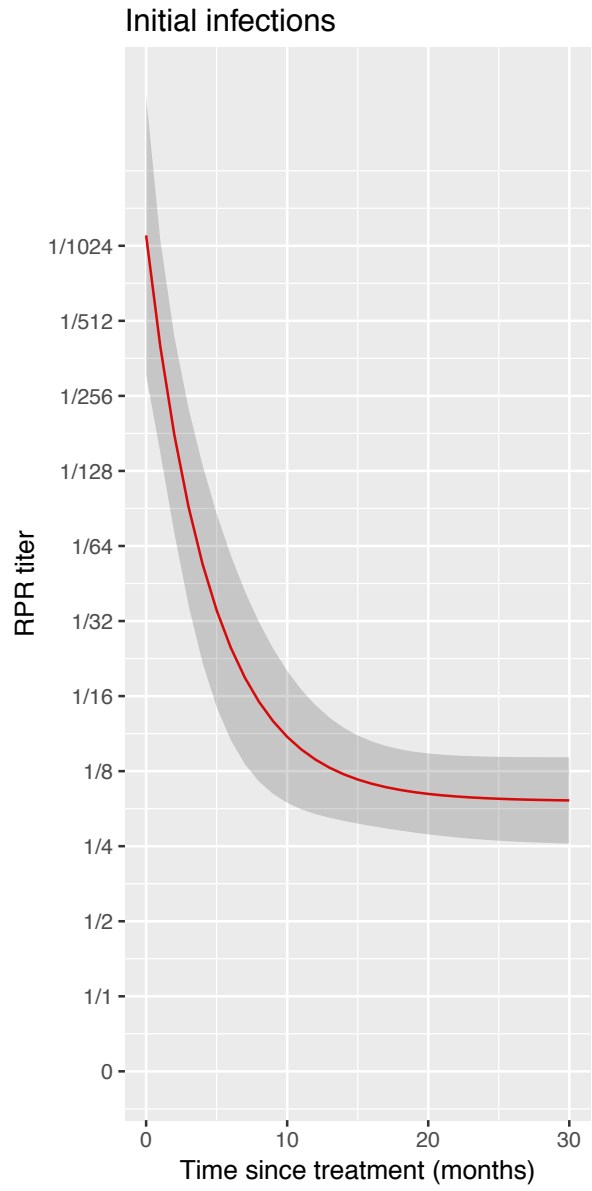
Initial RPR 1/128



Initial RPR 1/256



Initial RPR 1/512



Initial RPR 1/1024

References

1. Chen CY, Chi KH, George RW, Cox DL, Srivastava A, Rui Silva M, et al. Diagnosis of gastric syphilis by direct immunofluorescence staining and real-time PCR testing. *J Clin Microbiol.* 2006;44(9):3452-6. doi: 10.1128/JCM.00721-06. PubMed PMID: 16954299; PubMed Central PMCID: PMC1594693.