Improving care for red blood cell alloimmunized pregnant women

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Chapter 5

Predicting anti-Kell mediated hemolytic disease of the fetus and newborn, diagnostic accuracy of laboratory management.

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Abstract

Background: There is controversy on critical cut-off values of laboratory testing to select pregnancies at increased risk for anti-Kell (K) mediated HDFN (hemolytic disease of the fetus and newborn). Without early detection and treatment, Anti-K mediated HDFN may result in progressive fetal anemia, fetal hydrops, asphyxia and perinatal death.

Objective: We aimed to determine the value of repeated anti-K titer determination and biological activity measurement using the antibody-dependent cellular cytotoxicity (ADCC) test determination in the management of pregnancies at risk for anti-K mediated HDFN.

Study design: Retrospective cohort study of pregnancies with anti-K and a K-positive fetus, identified from January 1999 until April 2015. Laboratory test results and clinical outcome were collected from the Dutch nationwide screening program and the national reference center for fetal therapy in the Netherlands, the Leiden University Medical Center. Diagnostic accuracy (ROC-curves, sensitivity, specificity, positive and negative predictive values) of anti-K titers and ADCC test. The relationship between the titer and ADCC measurements and the two foregoing measurements were computed with a Pearson product-moment correlation coefficient.

Results: In a 16 year unselected cohort, representing screening results of 3.2 million pregnancies resulting in life births in the Netherlands, we identified 1,026 K-immunized pregnancies. 93 pregnant women had anti-K and a K-positive child, without other red cell alloantibodies. Forty-nine children (53%) needed intrauterine or postnatal transfusion therapy. The first anti-K titer showed already a high diagnostic accuracy with an AUC of 91%. The optimal cut-off point for the titer was 4 (sensitivity 100% (91-100; 95% CI), specificity 27% (15-43 95% CI) and positive predictive value 60% (49-71%). The ADCC test was not informative to select high-risk pregnancies. Linear regression showed no significant change during pregnancy, when antibody titer and ADCC test results were compared with every two foregoing measurements ($p<0.0001$).

Conclusion(s): Early determination of the anti-K titer is sufficient to select pregnancies at increased risk for HDFN with need for transfusion therapy. If the K status of the fetus is known to be positive, a titer of 4 or higher can be used to target intensive clinical monitoring.

Keywords: alloimmunization, anti-K, diagnostic accuracy, hemolytic disease of the fetus and newborn (HDFN), intra-uterine blood transfusion, laboratory tests, red blood cell antibodies, screening program.

Introduction

Hemolytic Disease of the Fetus and Newborn (HDFN) is caused by red blood cell (RBC) antibodies developed by the mother and transferred to the foetus.(10, 25) Kell (K) alloantibodies are second to RhD alloantibodies in importance as the cause of severe HDFN.(10, 25) K alloantibodies cause hemolysis of fetal erythrocytes and also inhibit the fetal erythropoiesis.(135-137) Without treatment, HDFN may result in progressive fetal anemia, fetal hydrops, asphyxia and perinatal death. (138) After birth, neonatal hyperbilirubinemia may lead to 'kernicterus', cause of neurodevelopmental impairment with athetoid cerebral palsy, hearing problems and psychomotor handicaps.(3, 5-8, 139, 140) Even though the incidence of fetal alloimmune hydrops has declined in the last decades,(141) this condition is still a well-known risk factor for adverse perinatal and long-term outcomes.(12) Severe anti-K-mediated HDFN may develop early in pregnancy, and often presents with hydrops before 20 weeks gestation.(12, 137, 142) Postnatally, anti-K-mediated HDFN is characterized more frequently by anemia than by hyperbilirubinemia, compared with HDFN caused by anti-D or other type of Rh alloantibodies.6

Red blood cell (RBC) alloimmunization should ideally be detected early in pregnancy upon routine RBC antibody screening. In most centers, to identify pregnancies at risk for severe HDFN, the titer of clinically relevant RBC alloantibodies is determined. (10, 21, 26, 139) If the titer is above a certain threshold, patients are referred to a maternal-fetal medicine center for close surveillance and, if needed, for fetal or neonatal treatment. (21, 26) High-risk pregnancies are monitored with ultrasound and Doppler middle cerebral artery peak systolic velocity (MCA-PSV) measurements, to predict the presence of fetal anemia.(18, 29, 143) Severe fetal anemia can be successfully treated using intrauterine transfusions (IUT). Neonates may require phototherapy or neonatal (exchange) transfusions.(144)

In the Netherlands, fetal K (Kell) genotyping is performed with cell-free fetal DNA isolated from maternal plasma.(22) K-alloimmunized pregnancies with a K-positive fetus are monitored by serial antibody titer measurements and by the Antibody Dependent Cellular Cytotoxicity (ADCC) bioassay, a monocyte based assessment of the destructive capacity of the antibodies.(58, 145, 146) However, there is still controversy on which critical titers and ADCC cut-off levels indicate a high risk for anti- K-mediated HDFN.(21, 26, 61, 147-150)

The aim of this study was to assess the performance of anti-K titer and ADCC measurements in K-alloimmunized pregnancies with a K-positive fetus, to predict severe HDFN requiring transfusion therapy.

Methods

Setting and Prevention program in the Netherlands

In the Netherlands, all pregnant women are typed for ABO, RhD and Rhc blood group antigens and screened for RBC antibodies at the first trimester booking visit. All screen-positive samples are sent to one of two national reference laboratories for confirmation and determination of the antibody specificity. These laboratories are Sanquin Diagnostics, Amsterdam (90% of the pregnant population) and the Special Institute for Blood Group Investigations (BIBO), Groningen (10% of the pregnant population). When clinically relevant RBC antibodies are detected, i.e. antibodies with the potency to destroy fetal RBC's, the father of the fetus is typed for the cognate antigen(s). In case the father is antigen-positive, or his type is not known, non-invasive fetal typing with cell-free fetal DNA isolated from maternal plasma is offered (for *RHD*, *RHC*, *RHc*, *RHE* and *K*), since 2004. (22) If the fetus is antigen-positive, serial testing (starting with every four weeks, from 24 weeks every three weeks, from 36 weeks every two weeks) of maternal antibody titers and the ADCC test is performed. Following current Dutch guidelines, a K-antibody titer ≥ 2 and/or an ADCC-test result ≥30% indicate a substantial risk for K-mediated HDFN, and the fetus will be weekly or every two weeks monitored with MCA Doppler measurements.(26) Laboratory follow-up is stopped if these thresholds are reached. Severe fetal anemia is treated with intrauterine transfusion(s) (IUT's) at the Leiden University Medical Center (LUMC), which is the national Dutch reference center for fetal therapy. The threshold for suspected severe fetal anemia requiring IUT was 1) a MCA-PSV of 1.5 multiples of the median for gestational age (MoM), detected by Doppler measurement, and/or 2) the presence of other signs of anemia at ultrasound examination (cardiomegaly, ascites, hydrops), or 3) amniotic fluid delta optical density measurements reaching the upper part of Liley's zone II or zone III (only in the early years of this study). (29, 151)

Laboratory testing

Both reference laboratories assess antibody titers, in phosphate-buffered saline by doubling dilutions, with the indirect antiglobulin test (IAGT), using an anti-IgG reagent and heterozygous K-positive RBCs.(152)

The ADCC test, as described by Engelfriet and Ouwehand, is only performed at Sanquin Diagnostics in Amsterdam.(58) Fetal K typing is also only performed at Sanquin Diagnostics.(22)

Study design

We performed a retrospective cohort study, including all pregnancies diagnosed with anti-K in the Netherlands, between January 1st 1999 and April 1st 2015. All K-immunization cases were identified at the two national reference laboratories. Women with K alloimmunization and antibody titers ≥2 and/or ADCC test results > 30% were usually referred to the LUMC for monitoring or treatment. All these cases could therefore also be identified in the LUMC database. We only included pregnancies with a K-positive fetus.

Outcomes

The primary outcome was the diagnostic accuracy (sensitivity, specificity and predictive values) of antibody titers and ADCC tests to predict severe K-mediated HDFN, which was defined as the need for intrauterine or postnatal transfusion.

Data collection

We collected the results of laboratory monitoring during pregnancy from Sanquin Diagnostics and data concerning clinical monitoring and IUT treatment during pregnancy, from the LUMC databases. Neonatal outcome data on treatment with blood transfusion(s) or phototherapy during the first three months of life were extracted from their medical files, by contacting the obstetric care provider, the pediatrician or the local hospital laboratories.

Analysis

Categorical variables were described as number and percentage and continuous variables by median and interquartile range P25-75%. To establish the optimal cut-off for antibody titer and ADCC test results, Receiver Operating Characteristics (ROC) curves were constructed for both the first and the highest measurement. Subsequently, the sensitivity, specificity and positive and negative predictive values for the prediction of fetal and neonatal hemolytic disease were calculated with 2x2 tables for different cut-off levels. To determine the best interval between consecutive titer and ADCC measurements, in order to adequately predict severe HDFN, a linear regression analysis (Pearson product-moment correlation coefficient) was performed. All analyses were performed with SPSS Statistics (version 23).

Ethical considerations

Clinical data were provided by the health care professionals as part of the quality evaluation of the routine laboratory monitoring of RBC alloantibody-complicated pregnancies The data were stored according to the Dutch established codes of conduct for responsible use of patient material and data, as approved by the Leiden University Medical Center. Ethical approval was not necessary according to the Dutch law on medical scientific research involving human subjects and according to the rules published by the Central Committee on Research involving Human Subjects (http://www.ccmo.nl/nl/niet-wmo-onderzoek).

Results

Study population

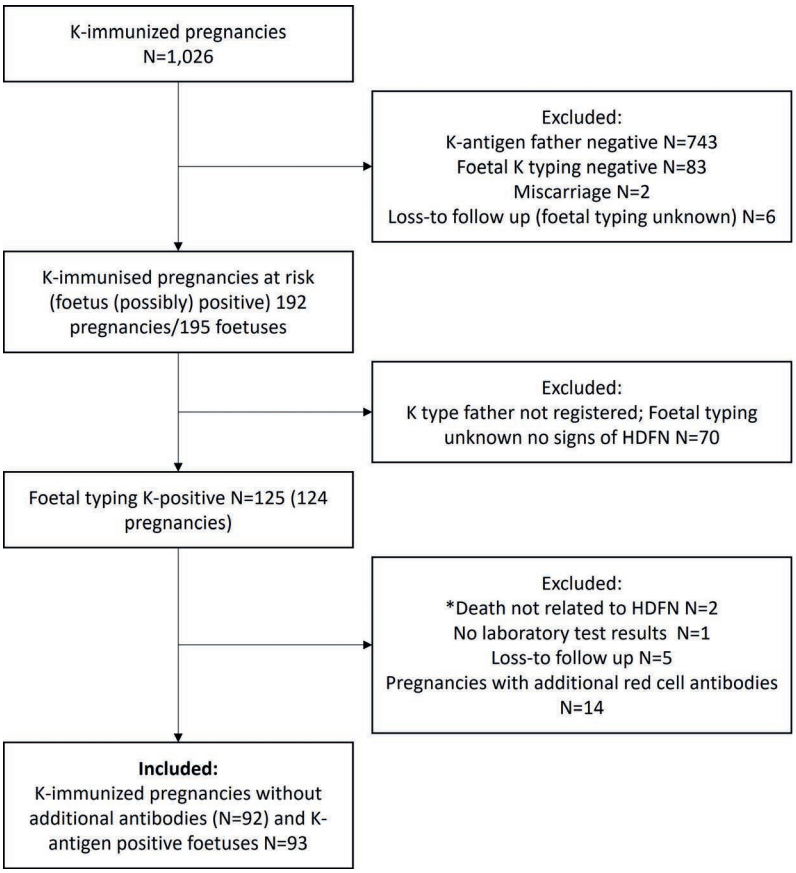
In 16 years, 1,026 K-immunized pregnancies were identified, including three pair of twins (Figure 1). After exclusion of pregnancies with K-negative fathers (n=743) and/or K-negative fetuses/children (n=83), miscarriages (<16 weeks, n=2) and loss-to follow up (n=6), 192 pregnancies with 195 K-positive fetuses remained for analysis. In another 70 pregnancies, the K type of the father and that of the child were unknown (after 2008 all fetal K status were known), but the absence of any sign of HDFN could be confirmed in all cases. After exclusion of these pregnancies, 124 pregnancies with 125 K-positive fetuses remained.

Another seven of these 125 fetuses were excluded from our analysis, because of perinatal death, clearly not related to K-immunization (n=2) or unknown neonatal outcome (n=5). One case with severe HDFN (hydrops) detected late in pregnancy was excluded, because of lack of laboratory data. Furthermore, we excluded 24 cases with additional red cell alloantibodies, which may have contributed to the severity of HDFN. The remaining 93 (92 pregnancies) K-positive fetuses were used in our diagnostic accuracy analysis.

Fifty-six percent (49/93) of children received either intrauterine (48/93; 52%) or neonatal (1/93; 1%) transfusion, whereas the remaining 47% (44/93) had no signs of HDFN or was only treated with phototherapy . There were three cases with two pregnancies, all women had in all pregnancies titers above 4.

There were three cases of perinatal death related to the K-immunization (titers variating from 128-1024). One case was closely monitored every two weeks with ultrasound. At 23 weeks the fetus was unexpectedly found to be hydropic, with an MCA Doppler result indicating fetal anemia. Fetal demise occurred just before the first IUT could be given. One case was a very early onset of fetal anemia with hydrops detected at 15+3 weeks of gestation. The fetus died at 16 weeks; intrauterine transfusion was not performed because of the poor condition and prognosis at this early stage of the pregnancy. In the last case, the fetus was initially predicted to be K-negative with non-invasive fetal K antigen typing, at 12 weeks of gestation. Awaiting the repeated and definitive K-typing result, clinical monitoring was not performed and the fetus died at 18 weeks of gestation. At 19 weeks ‘gestation, non-invasive fetal K typing showed the fetus to be K-positive.

Figure 1: *Flowcharts of inclusion K-immunized pregnancies with a K-antigen positive fetus.*



**Causes of death not related to HDFN were: one fetus had growth restriction in combination with dimorphism and died intra-uterine and one neonate had a congenital neuro-muscular disorder and died 7 days post-partum.*

The median gestational age at the first laboratory testing was 14 weeks (P25-P75: 13-18 weeks). The median gestational age at the last measurement of titer and ADCC test was 26 weeks (P25-75: 18-35 weeks). The median first K-titer was 64 (P25-75: 8-256) and the highest median titer was 128 (P25-75: 16-256). The median first ADCC was 35% (P25-75: 10-57.5%) and the highest median ADCC was also 35% (P25-75: 15-65%). The median number of days from the last test to the first IUT or to delivery was 26 days (P25-P75: 9-77 days). The first IUT was performed in week 24 (P25-75: 22-28 weeks). The median number of laboratory tests performed per pregnancy was 5 (P25-75: 3-8).

Table 1.

First titre Cut-off	Positive tests	Need for transfusion					
		True positive	*Missed HDFN cases	Sens % (95%-CI)	Spec % (95%-CI)	PPV % (95%-CI)	NPV % (95%-CI)
≥2	81	49	0	100 (91-100)	27 (15-43)	60 (49-71)	100 (70-100)
≥4	77	49	0	100 (91-100)	36 (23-52)	64 (52-74)	100 (76-100)
≥8	70	47	2	96 (85-99)	48 (33-63)	67 (55-78)	91 (70-98)
≥16	62	47	2	96 (85-99)	66 (50-79)	76 (63-85)	94 (77-99)
≥32	57	45	4	92 (80-97)	73 (57-85)	79 (66-88)	89 (73-96)
≥64	50	43	6	88 (75-95)	84 (70-93)	86 (73-93)	86 (71-94)
≥128	43	38	11	78 (63-88)	89 (75-96)	88 (74-96)	78 (64-88)

Number of positive tests, sensitivity, specificity and predictive values of K-mediated pregnancies without additional antibodies to predict the need for transfusion by cut-off first titre (N=93).

**Cases with necessity for transfusion therapy that would be missed when cut-off used.*

Diagnostic accuracy of the K-antibody titre and ADCC test

The ROC curves for respectively the first and the highest anti-K titer, correlating with severe HDFN, with need for transfusion therapy (n=93) The Area Under the Curve (AUC) for the first measured K antibody titer to predict the need for transfusion therapy was 0.917, for the highest titer during pregnancy the AUC was 0.906. We defined the optimal cut-off point at a sensitivity of 100% (91-100 95% CI) and combined with the highest specificity of 27% (15-43, 95% CI). Thus, an optimal cut-off for the first and highest titer was assessed at 4 (table 1).

The AUC for the highest ADCC test result was 0.843. If a sensitivity of 100% was taken, the optimal cut off value appeared to be below the first test outcome of ADCC <10% (data not shown). Therefore, additional ADCC testing seems to be not informative for the prediction of severe HDFN (data not shown).

Linear correlation between consecutive measurements of titre and ADCC test

Since the AUCs for the highest titer and the first titer hardly differed, we investigated whether the titer and ADCC test results changed significantly during pregnancy. Linear regression showed no significant change, when antibody titer and ADCC test results were compared with every two foregoing measurements. A Pearson product-moment correlation coefficient was computed to assess the relationship between the titer and ADCC measurements and the two foregoing measurements (for scatterplots see appendix 1 a,b,c,d). Overall, there was a strong correlation between titer and ADCC measurements with the two foregoing measurements. The small, non-clinical relevant difference between the measurements is explained for 94% and 91% (titer) and 87% and 84% (ADCC) by the two foregoing measurements ($p<0.0001$).

Comments

Main Findings

In a 16 year unselected cohort, representing screening results of 3.2 million pregnancies resulting in life births in the Netherlands, we identified 93 pregnancies complicated by the presence of anti-K in the presence of a K-positive fetus. We determined that, if the K status of the fetus is positive, an anti-K titer of 4 identifies all cases with a high risk for severe HDFN, defined as the need for intra-uterine or postnatal transfusions. Test results of both titer and ADCC-test did not change

significantly during pregnancy. The first titer appeared to have the highest power to predict the necessity of transfusion therapy in K-alloimmunized pregnancies.

Strengths and weaknesses

To our knowledge this is the first large registry-based cohort study, including an unselected complete population of K-alloimmunized pregnant women with a K-positive child. Most other studies included a selected group of women with an increased risk for severe HDFN, for example women referred to a regional or national referral center.(147-149)

In our study, 93 out of 1,026 (9%) of the K-immunized pregnancies were considered as at risk for HDFN (fetus K-positive) and included in the analysis. A weakness of our study is that the K status was not known for all fetuses; yet we think no severe cases of HDFN with need for intra-uterine transfusion were missed as they would have been referred to the LUMC.

Clinical implications

Overall, we observed in this unselected population, that over 50% of K-positive fetuses of K-alloimmunized mothers need either intrauterine or postnatal transfusion therapy. At a cut-off of 4 for the first titer the specificity is 27% and the positive predictive value for transfusion therapy is 60%. This relatively high positive predictive value implicates that transfusion therapy is needed in two out of three K-positive children of mothers with a K titer of 4 or above, and may be an argument to accept this relatively low cut-off titer. In order to fine tune the selection of high-risk cases, it would making it worthwhile to add fetal K typing to the diagnostic algorithm. With higher cut-off titers, for example 16, still 96% of severe cases will be followed, raising test specificity to 66% and the positive predictive value to 76%. With this cut-off titers of 16 the number of missed cases of severe HDFN is two.

Our proposed optimal cut-off point of the anti-K antibody titer of 4 is only one dilution step lower than suggested by Moise et al., who proposed a threshold of 8. It is also lower than McKenna et al.[147] who proposed a threshold of 32, based on a smaller cohort with eight cases of severe HDFN, all with titers of >=32. In contrast, Leggat et al., including 16 K-positive fetuses, and our previous study, as reported by Van Wamelen et al. including 41 K-positive fetuses, reported one case each needing intrauterine transfusion therapy at anti-K titers of 2. (148, 149) These studies thus support our finding that also a low anti-K titer can be responsible for a severe course of anti-K-mediated HDFN. However, in our study only 16 pregnancies of 93 cases

had titers below 4, we didn't find severe cases of HDFN in this group. Therefore K-mediated HDFN with need for transfusion therapy in cases with titers below 4 is very rare.

Although titer measurements can vary between laboratories, also with established techniques, in general a comparison be made with one-fold dilution difference between observers and between laboratories. It is advised to use an indirect antiglobulin test without additives.(152) Non-invasive fetal K-typing with cell-free DNA isolated from maternal plasma is not available in all countries. Fetal K-typing can be performed with DNA obtained via amniocentesis. Amniocentesis is an invasive procedure, with risks for the pregnancy and a possible rise in anti-K titers. Therefore, it should be considered, if non-invasive fetal K typing is not possible via a reference laboratory, if close monitoring of anti-K complicated pregnancies with MCA-Doppler can be used for timely detection of the occurrence of fetal anemia.

We observed that the monocyte-based ADCC test was not suitable to accurately select high-risk K-alloimmunized pregnancies. This might be due to the pathogenesis of anti-K-mediated HDFN, in which both the suppression of erythropoiesis and hemolysis of fetal RBC may be of importance.(135, 136, 153) Recently, it was also shown that the glycoprofile of alloantibodies may influence antibody pathogenicity and therefore a putative diagnostic marker.(153) Therefore, it may be that other type of bioassays, testing such antibody characteristics, may improve test specificity. (146, 154, 155, 27)

A first step in the diagnostic algorithm, that we currently use, is non-invasive fetal K typing with cell-free DNA isolated from maternal plasma.(156) Prediction of K-negativity warrants a high sensitivity of this PCR-based testing and the confirmation on sufficient levels of fetal DNA; also in our series early prediction of K-negativity was incorrect once in week 12 of pregnancy.(157) Since cell-free fetal DNA levels raise in the first trimester of pregnancy, for fetal K typing a conclusive result can in general be provided around week 18 of pregnancy.(22)

Conclusion

To select pregnancies with an increased risk for anti-K-mediated HDFN requiring frequent monitoring to detect fetal anemia, determination of the anti-K titer once early in pregnancy is sufficient. The optimal cut-off value is a titer of 4. Following the detection of anti-K, fetal K-typing, preferably using a non-invasive method, is an important step in efficient management. In pregnancies with an anti-K titer of 4 or higher and a positive fetus, 60% of fetuses or neonates requires transfusion therapy. Since the ADCC test is not useful in the prediction of fetal hemolysis in the presence of an anti-K we recommend discontinuing its use for these pregnancies.

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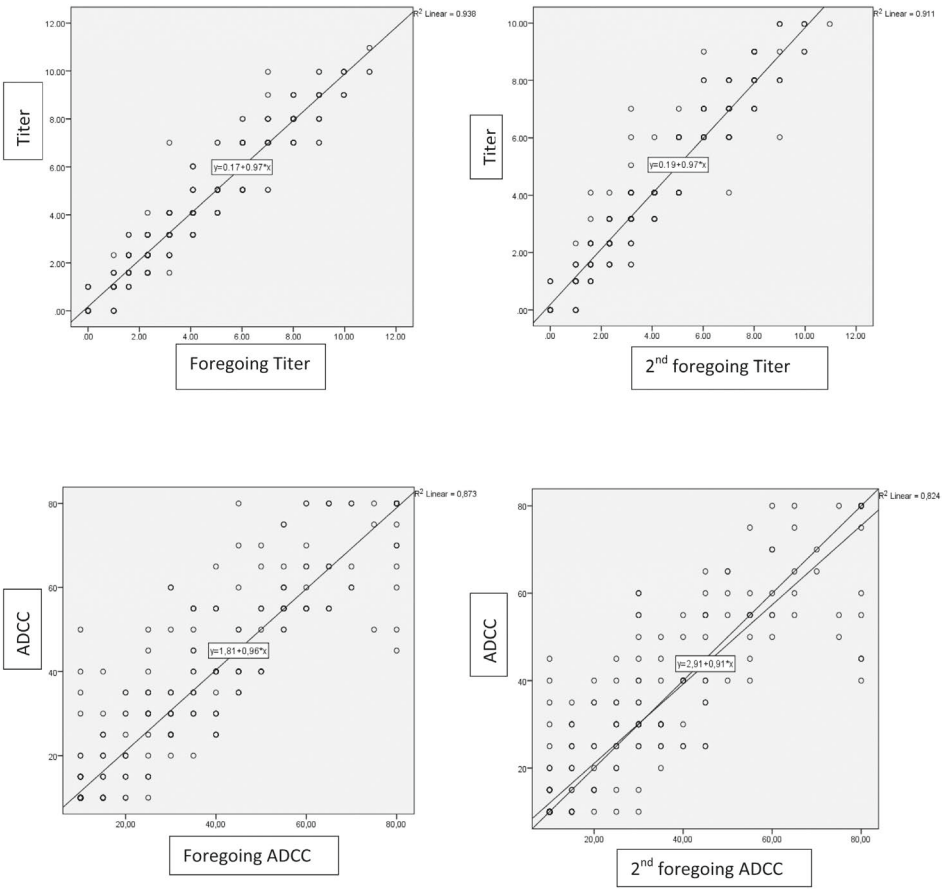

Appendix 1: *Scatterplots of relation between titer and ADCC measurements with the two foregoing measurements.*