SUMMARY

Epstein-Barr virus (EBV) is a common infectious agent with unique biological properties that, may lead to several benign and malignant diseases upon interaction with host or environment derived co-factors. Nasopharyngeal carcinoma (NPC) shows incidence of less than 1/100,000 western countries and Japan, but has intermediate (1-6/100.000) to high (>6/100.000) incidence in most of South-East Asia, China and North-Africa. Undifferentiated type carcinoma (WHO type III), the most prevalent form of NPC, has almost 100% correlation with EBV infection, with lower frequency in WHO type II (70%) and type I (<10%). The EBV-NPC association was shown by EBV clonality in the tumor and active expression of viral genes in all tumor cells. Furthermore, in peripheral blood of NPC patients elevated EBV-DNA levels and aberrant antibody responses to EBV proteins were detected.

Most NPC patients appear in clinics at late stage of malignancy, requiring complicated therapy, having low cure rate, and being subject to progression. Biopsy is the main diagnostic tool to determine the malignancy status. In addition, NPC can be identified by elevated titer of IgG or IgA to EBV encoded EBNA1, EA and VCA proteins in serum by means of indirect immunofluorescence techniques (IFA). However, IFA is a laborious technique, with subjective interpretation and not suitable for mass testing. ELISA is developed to replace IFA, allowing a simpler, more sensitive, and more standardized test system that may be used for high-through-put testing. However, this method requires well-defined and restricted number of EBV-antigens to mix in one well, in order to measure IgG/ IgA reactivity to multiple EBV antigens in NPC. Recently, quantification of EBV-DNA viral load from circulation has been proposed to be applied for diagnosis, monitoring therapy, and predicting relapse. However, the application of EBV-DNA quantification for routine clinical practice in developing countries is impeded by high costs, and infrastructural and logistical problems.

Within this thesis we describe the development of IgA-EBV based ELISAs and EBV-DNA viral load testing aiming for improved NPC diagnostic tools in Indonesia. First we analysed the molecular complexity underlying IgG and IgA anti-EBV antibody responses in NPC patients. We subsequently explored types of EBV antigens to be used in a one-step IgA-ELISA format. Thirdly, we applied real time-PCR for quantification of EBV-DNA in whole blood and nasopharyngeal (NP) brushings. NP-brush analysis provides a non-invasive and extremely useful method for demonstrating presence of NPC in situ in the nasopharyngeal space and may replace the invasive and painful biopsy as diagnostic procedure. We propose a combination system of serology and molecular assays for screening of people at high-risk of NPC, as well as those of at early stage of NPC. For daily-clinical and field application, we also suggest simpler sampling tools without affecting sensitivity and specificity of the systems.

In **chapter 2** we explored molecular diversity of IgG/ IgA responses in healthy persons and NPC patients from high, median, and low endemic area. We found that healthy individuals and NPC patients from different endemic areas share characteristic IgG and IgA response-patterns to EBV. Healthy EBV carriers show a restricted IgG response to EBNA1 and VCA-p18, and occasionally to VCA-p40 and ZEBRA, whereas they have a negative or weak IgA response to EBV antigens. On the other hand, NPC subjects showed stronger and more diverse IgG and IgA responses toward different types of EBV antigens, indicating different antigenic trigger. The individual IgG and IgA responses did not correlate with parallel IFA titration results. We were able to explore the diagnostic value of individual EBV proteins comprising the EA and VCA complex, which was impossible to obtain by IFA techniques. IgG responses to EBV showed to correlate with stage of malignancy and could be used to predict relapse. Although IgG-EBV pattern analysis by immunoblot proved to be a valuable diagnostic and prognostic test, this method still requires cell culture facility for antigen production, and is subjective in interpretation. On the other hand, this method allowed the selection of defined EBV proteins suitable for ELISA development, instead of using complex antigen mixtures as in IFA. Purified antigens from EBV-infected B cells culture, recombinant EBV proteins, or synthetic peptides can be used in an ELISA format to detect antibody responses specific for NPC patients.

In **chapter 3**, we explored the use of two synthetic peptides in an IgA-ELISA format. The EBNA1 and VCA-p18 synthetic peptides were derived from a combination of immundominant epitopes defined by PEPSCAN analysis and produced as long (approx. 60 AA) multi-epitope peptides. These synthetic peptides were used alone or in combination and used for IgA detection (IgA-EBV ELISA). When compared to IgG-EBV ELISA, we found that IgA-ELISA well-discriminated healthy and NPC populations. This supports the statement that IgA response to EBV is a hallmark of NPC. The IgA-EBV ELISA reactivity did not correlate with stage of disease due to limited numbers of early stage NPC patients. However, this system excluded acute infectious mononucleosis (IM) and chronic EBV. This IgA-EBV based ELISA shows to have sensitivity and specificity, PPV and NPV of 85.4, 90.1, 78.7, and 93.9% respectively, higher to those of IgA-ELISA with single antigen (note: a recently modified method using a high-binding ELISA plate yielded even higher sensitivity to about 90%; unpubl. data). Similar diagnostic values were found when the IgA-EBV-ELISA tested in a Chinese NPC population from Hong Kong, showing sensitivity and specificity, PPV and NPV of 91.1, 81.5, 95.6, and 89.3 % These data illustrate the diagnostic potential of the IgA-EBV ELISA to be applied in both Indonesian and Chinese populations. The synthetic peptide based EBV-IgA ELISA offers a cheap, sensitive and specific tool for NPC diagnosis, suitable for standardization and large-scale use. Therefore we propose to use this method for NPC screening and routine diagnosis in Indonesia.

NPC associates with humoral immune responses to multiple EBV antigens. The peptide-based IgA-ELISA described above still missed some proven NPC cases, requiring further optimization. Therefore, we re-explored the EBV molecular diversity of Ab-responses in biopsy-confirmed NPC patients who showed a negative reaction in the peptide-based IgA-EBV ELISA, to identify other potential antigen(s). We also analysed Ab-reactivity to various individual recombinant and peptides based alternative EBV antigens, but none gave satisfactory results to improve the IgA-EBV ELISA system (data not shown). **In chapter 4**, the IgG immunoblot strip analysis shows 100% reactivity to VCA-p40 (BdRF1) and VCA-p18 (BFRF-3) and less to other EBV antigens. Previous and unpublished data had shown that PEPSCAN analysis was not able to identify immunodominant (linear) epitopes of BdRF1. Therefore it was concluded that VCA-p40 epitope recognition may be more directed to conformational than sequence-based domains. A recombinant protein was made by fusion of full-length BdRF1 with BFRF3-encoding the dominant VCA-p18 epitope and expressed in *E.coli*. Purification of this recombinant protein was done by metal affinity chromatography via a histidine-tag at one end of the recombinant protein. By using high salt concentration and gradual increase of imidazole, we were able to purify the VCAp40+18 protein, yielding a 55kDa protein. Expression levels and purification efficiency are still low, and need to be optimized further. Nevertheless, we were able to use the purified recombinant protein IgA-VCAp40+18-ELISA (**IgA-VCA ELISA**) in a selected-restricted NPC panel with negative and positive IgA-EBV-ELISA. The IgA-VCA ELISA showed to detect 63.64% (14/22) and 95% (19/20) samples that were negative and positive in peptide-based IgA-EBV ELISA, respectively. When added to the overall NPC panel already tested with IgA-EBV ELISA (n=562), the IgA-VCA ELISA may increase the sensitivity from about 90% to 96.26%. The VCAp40+18 is proposed to combine

with the EBNA1 synthetic combi-peptide in one-well ELISA to produce a high sensitive NPC serodiagnostic tool. As an addition, the VCAp40+18 recombinant protein also showed a potential use for diagnosis of acute infectious mononucleosis.

The application of IgA-EBV based ELISA for field-screening should be accompanied by development of suitable, simple blood sampling method. In **chapter 5**, we explored the use of dried-blood filter paper sampling (DBS) with blood taken directly from finger-prick or from vacutainer-tube. The serum eluted from DBS showed to yield similar OD values as fresh or frozen plasma when tested in IgA-EBV ELISA format, with r^2 about 0.9. The only limitation is that sufficient sample volume (3 large drops; ~100ul) should be collected on the filter paper. This chapter also demonstrated the use of Whatman#3 filter paper as an alternative to the standard S&S#903 sampling paper. DBS showed to retain reactive IgG/ IgA antibodies over a long period of time when stored in dry-cold room. The IgG/ IgA retaining capacity was less when DBS was stored at higher temperatures, but leaving enough time for DBS to be "surface" mailed at ambient temperatures to diagnostic laboratory, without additional treatment. The DBS proved to be an alternative to fresh blood sampling, with finger prick offering a "friendly" and less invasive sampling method, combined with easy handling, transportation, and storage; thus, suitable for mass-sampling.

In **chapter 6**, we explored the application of unfractionated whole blood for EBV-DNA quantification by a standardized LightCyler-PCR (LC-PCR), using a highly conserved EBNA1 gene segment as amplification target. EBV-DNA quantification is based on serial dilution of a known quantity of EBV genome as standard. Unfractionated whole blood is chosen as it represents all blood compartments. The silica-based DNA/RNA-isolation method showed practical advantages (simple procedure, not needing expensive equipment), and only required small volume of blood. It allows parallel isolation of DNA and RNA, while eliminating PCR-inhibitors. Whole blood LC-PCR showed higher positive rate when applied to a primer producing 99bp (85.9%) than 213bp (72.5%) of PCR amplimer. This may relate to the finding that the majority of circulating EBV-DNA in NPC patients is fragmented, probably deriving from apoptotic tumor cell fragments. We found that only limited number of NPC patients had significant elevated EBV-DNA load in whole blood, limiting its use as primary diagnostic marker. Yet, EBV-DNA load in blood may be useful for therapy monitoring (M. Adham, in progress). The detection of EBNA1 mRNA in blood of some NPC samples indicated the presence of circulating B-cells. The absence of BARF1-RNA in whole blood indicates absence of circulating tumor cells. Detection of EBV-DNA in blood yielded low sensitivity values, thus being of limited use for primary NPC diagnosis.

We further explored the use of LC-PCR for analyzing EBV-DNA viral load in brush samples collected at the primary site of tumor development, being the nasopharyngeal epithelium. In **chapter 7,** we described the use of non-invasive nasopharyngeal brushing (NP brush) to obtain cells from the tumor, and quantify the EBV-DNA and RNA expression pattern. This system showed sensitivity, specificity, PPV and NPV values of 90, 98, 97, and 91% respectively, much higher than those of unfractionated whole blood. NP brush can be applied more frequently for tumor inspection as it is a relatively easy and less invasive method compared to biopsy procedure. Highly positive results were found in cases with parallel negative whole blood EBV-DNA loads. NP brush offers an *in-situ* DNA measurement, reflecting direct EBV biological activity. Additionally, the NASBA technique was able to detect presence of EBNA1 LMP2 and BARF1 mRNA in 88% of brush samples (indicating presence of intact tumor cells). BARF1 RNA is expressed only in EBV-related carcinoma, and should be further explored as candidate NPC marker.

Finally, we propose to combine peptide-based IgA-EBV ELISA and measurement of EBV-DNA viral load or BARF1-RNA in NP brushings for screening and primary diagnosis of NPC in high risk groups. People at risk of NPC include individuals with (1) certain chronic complaint(s) in head and neck area, (2) environmental (dietary and non-dietary) risks, or (3) with familial NPC. These should have their blood tested for IgA-EBV by ELISA. Finger-prick DBS may be a good tool to obtain the blood sample. High IgA-EBV reactivity should preferably be confirmed by NP brushing (accompanied with inspection by nasoendoscopy) to quantify the EBV-DNA or BARF1 RNA. Positive result for both serology and molecular tests should give a strong recommendation for further clinical inspection, possibly a biopsy and CT-scan to define the NPC location definitively. These approaches should be able to identify people at early stage of malignancy. Thus, medical intervention may be applied immediately. On the other hand, individuals with positive test results but with negative-clinical examination should be regularly monitored (each 6-12 months), at clinical and EBV-based laboratory level, because positive results suggest (clinically obscure) early stage nasopharyngeal tumor process. High and increasing IgA-EBV and EBV-DNA viral load values should raise awareness of tumor progression. For primary diagnostic use, the IgA-EBV ELISA may be used in combination with EBV-DNA viral load from the nasopharynx. The dynamics of IgA-EBV and EBV-DNA viral load during and after chemoradiation treatment may be used to predict remission (declining values) and persistent disease or relapse (increasing values). Elevated EBV-DNA viral load in blood reflects tumor cell apoptosis and may be useful to monitor therapy efficacy.

The direct link between aberrant EBV activity and NPC pathogenesis allows the use of defined (anti-)viral markers for diagnosis, prognosis and therapy monitoring. The molecular and immunological tests developed in this thesis may contribute to a more wide-spread application of this concept in developing countries, improving early-stage NPC detection and cancer-related health care.