Curtin Medical School

Investigating the role of inflammatory and neuronal repair genes in the neurological sequelae of HIV infection

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Ethics Declaration

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

The research presented and reported in this thesis was conducted in accordance with the National Health and Medical Research Council National Statement on Ethical Conduct in Human Research (2007) – updated March 2014. The proposed research study received human research ethics approval from the Curtin University Human Research Ethics Committee (EC00262), Approval Numbers:

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Abstract

HIV-associated sensory neuropathy (HIV-SN) is a debilitating condition affecting up to 60% of HIV+ individuals treated with antiretroviral therapy (ART) that included stavudine. As this prompted the withdrawal of stavudine from recommended regimens, studies of the prevalence, risk factors and underling mechanisms of HIV-SN need to be repeated. HIV-associated neurocognitive disorder (HAND) describes a spectrum of neurocognitive impairments that affect up to 70% of HIV+ individuals, even with effective ART. As the clinical pathology of HIV-SN and HAND involve inflammation and degeneration of neurons, investigation of genes affecting these pathways may illuminate critical mechanisms. This thesis assessed the role of single nucleotide polymorphisms (SNP) in the *TNF*-block (nine central MHC genes) and the *P2X*-block (*P2X7R*, *P2X4R*, *CAMKK2* and *ANAPC5*) in HIV-SN, and the *P2X*-block in HAND.

Chapters 2-4 describe associations between HIV-SN and *TNF*- and *P2X*-block SNP and haplotypes in South Africans and Indonesians. African participants were recruited and assessed for HIV-SN using the Brief Peripheral Neuropathy Screening Tool (BPNS) as they commenced stavudine-free ART and after 6-8 months by our colleagues at the University of the Witwatersrand. Indonesian participants stable on stavudine-free ART for >12 months were recruited and assessed using the BPNS by our colleagues at Universitas Indonesia. I genotyped participants for 64 SNP across both gene-blocks using the OpenArray platform, derived haplotypes using fastPHASE, assessed demographic/clinical risk factors, and derived optimal logistic regression models.

12% (9/75) of Africans had HIV-SN prior to commencing ART and a further 27% (20/75) developed HIV-SN in the following 6-8 months. 17% (35/202) of Indonesians had HIV-SN. Optimal *TNF*-block models associated the minor allele of rs4947324 (*NFKBIL1*) with lower rates of HIV-SN in Africans after adjusting for body weight, CD4 T-cell counts and prior tuberculosis (TB), and rs9281523 (BAT1[intron10]) with greater risk of HIV-SN in Indonesians after adjusting for CD4 T-cells and viremia (viral load >500). Haplotype analyses reflected these results and suggest rs4947324 and rs9281523 may be in linkage disequilibrium (LD) with a common causal variant. Optimal *P2X*-block models independently associated one *P2X7R* and three *CAMKK2* SNP with altered susceptibility to HIV-SN after adjusting for body weight, CD4 T-cells and prior TB in Africans. In Indonesians, three different *CAMKK2* SNP (in perfect LD), and one *P2X7R* and *CAMKK2* haplotype were independently associated with altered risk of HIV-SN after adjusting for CD4 T-cells and viremia. These results demonstrate that HIV-SN remains a common complication despite the discontinuation of stavudine and confirms a role for the *TNF*- and *P2X*-block in HIV-SN.

To further investigate the clear link between HIV-SN, a subset of Indonesians underwent assessment for large and small fibre neuropathy using nerve conduction (NC) and stimulated skin wrinkling (SSW) tests, respectively (Chapter 5). SSW and BPNS diagnoses did not align but both diagnoses associated with SNP in *CAMKK2.* NC did not, so SNP in *CAMKK2* may affect small fibres in HIV-SN.

Chapter 6 used confocal microscopy to visualise expression of three proteins encoded by the *P2X*-block (P2X7R, P2X4R and CaMKK2) in the epidermis of individuals with and without HIV-SN. P2X7R was expressed by cells in blood vessels of HIV-SN− donors but was rare in HC or HIV-SN+ donors. P2X4R was expressed by cells in blood vessels and by cells in the basal layer of the epidermis. Expression by cells in the epidermal basal layer appeared greatest in HIV-SN+ donors. CaMKK2+ cells were rare in HC. HIV-SN− donors appeared to have fewer CaMKK2+ cells than HIV-SN+ donors. CaMKK2+ cells were located close to dermal and epidermal nerve fibres.

Chapters 7 and 8 described associations between *P2X*-block SNP and neurocognitive impairment in HIV+ Indonesians (n=59). Participants were recruited and assessed for neurocognitive impairment as they commenced stavudine-free ART and after 3, 6 and 12 months by our colleagues at the Universitas Indonesia, and participants were genotyped for *P2X*-block SNP by our colleague in Perth. I derived linear regression models identifying five intronic SNP in *P2X7R* which influenced neurocognitive impairment in specific domains and stages of recovery on ART. rs504677 associated with lower executive function and motor speed Z-scores at all time-points. rs1653598 influenced executive function and motor speed Z-scores at 0-6 months. rs208296 associated with higher memory Z-scores at 0-6 months of ART. rs208307 associated with higher memory and executive function and rs11065504 with higher attention Z-scores at 0 and 12 months. Three additional SNP from *CAMKK2* (rs2686344, rs1653587, rs1718120) were linked with altered fluency and executive function outcomes after 12 months on ART. These results suggest *P2X7R* may influence HAND in a domain- and time-specific manner and *CAMKK2* may play a role in the recovery of HAND.

In conclusion, these result demonstrate that SNP within the *TNF*- and *P2X*-blocks do influence HIV-SN and HAND, implicating a role for the encoded proteins. Further investigations to identify causative alleles and the pathways involving the encoded proteins are warranted. These should address the roles of P2X-block genes in neuropathy and neurocognitive loss that is not associated with HIV.

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We acknowledge that Curtin University works across hundreds of traditional lands and custodial groups in Australia, and with First Nations people around the globe. We wish to pay our deepest respects to their ancestors and members of their communities, past, present, and to their emerging leaders. Our passion and commitment to work with all Australians and peoples from across the world, including our First Nations peoples are at the core of the work we do, reflective of our institutions' values and commitment to our role as leaders in the Reconciliation space in

Statement of Candidate Contribution

All work was performed by the author unless otherwise stated in the thesis

Jessica Gaff
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A/Professor Patricia Price
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Professor Peter Kamerman
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Research Output

Publications forming part of this thesis

Chapter 2
Gaff J, Octaviana F, Pillay P, Mbenda HGN, Ariyanto IA, Gan JA, Cherry CL, Kamerman P, Laws SM, Price P. TNF-Block Genotypes Influence Susceptibility to HIV-Associated Sensory Neuropathy in Indonesians and South Africans. Int J Mol Sci. 2020 Jan 7;21(2):380. doi: 10.3390/ijms21020380. PMID: 31936167; PMCID: PMC7014294.

Chapter 3
Gaff J, Pillay P, Cherry C, Laws SM, Price P, Kamerman P. The role of CAMKK2 polymorphisms in HIV-associated sensory neuropathy in South Africans. J Neurol Sci. 2020 Jun 15;416:116987. doi: 10.1016/j.jns.2020.116987. PMID: 32585444.

Chapter 4
Gaff J, Octaviana F, Ariyanto I, Cherry C, Laws SM, Price P. Polymorphisms in CAMKK2 associate with susceptibility to sensory neuropathy in HIV patients treated without stavudine. J Neurovirol. 2019 Dec;25(6):814-824. doi:10.1007/s13365-019-00771-w. PMID: 31309408.

Chapter 5
Yanuar Safri A, **Gaff J**, Octaviana F, Dewanto Setiawan D, Imran D, Cherry CL, Laws SM, Price P. Demographic and genetic associations with markers of small and large fibre sensory neuropathy in HIV patients treated without stavudine. J Acquir Immune Defic Syndr. 2020 Sep 7. doi: 10.1097/QAI.0000000000002503. PMID: 32925363.

Chapter 7
Gaff J, Estiasari R, Diafiri D, Halstrom S, Kamerman P, Price P. Neurocognitive outcomes in Indonesians living with HIV are influenced by polymorphisms in the gene encoding purinergic P2X receptor 7. Brain, Behavior, & Immunity - Health. 2021;13:100220.

Manuscripts submitted for publication which form part of this thesis

Chapter 6
Gaff J, Octaviana F, Jackaman C, Kamerman P, Papadimitriou P, Lee S, Mountford J, Price P. Epidermal expression of calcium/calmodulin-dependent kinase kinase 2 and purinergic receptor 7 and 4 in HIV-associated sensory neuropathy

Chapter 8
Gaff J, Estiasari R, Diafiri D, Pramana S, Djauzi S, Halstrom S, Price P. Polymorphisms in CAMKK2 may influence domain-specific neurocognitive function in HIV+ Indonesians receiving ART

Publications not forming part of this thesis

Appendix 1
Gaff J, Jackaman C, Papadimitriou J, Waters S, McLean C, Price P. Immunohistochemical evidence of P2X7R, P2X4R and CaMKK2 in pyramidal neurons of frontal cortex does not align with Alzheimer's disease. Exp. Mol. Pathol. 2021;120:104636.

Appendix 2
Gaff J, Halstrom S, Temple SEL, Baltic S, Kamerman P, Price P. Polymorphisms in P2X4R and CAMKK2 may affect TNFα production: Implications for a role in HIV-associated sensory neuropathy. Hum Immunol. 2018 Apr;79(4):224-227. doi:10.1016/j.humimm.2018.02.002. Epub 2018 Feb 8. PMID: 29428485.

Appendix 3
Mountford J, Octaviana F, Estiasari R, Setiawan DD, Ariyanto I, Lee S, **Gaff J**, Chew C, Jackaman C, Kamerman P, Cherry C, Price P. Ex-vivo expression of chemokine receptors on cells surrounding cutaneous nerves in patients with HIV-associated sensory neuropathy. AIDS. 2018 Feb 20;32(4):431-41. doi: 10.1097/QAD.0000000000001714. PMID: 29239897.

Appendix 4
Dmello DM, Ariyanto I, Estiasari R, Halstrom S, **Gaff J**, Lee S, Price P. Polymorphisms in IL10 may alter CD4 T-cell counts in Indonesian HIV patients beginning antiretroviral therapy. Hum Immunol. 2017 Apr;78(4):387-390. doi: 10.1016/j.humimm.2017.03.001. Epub 2017 Mar 2. PMID: 28263776.

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List of Abbreviations

ACTG BPNS	AIDS clinical trial group brief peripheral neuropathy screening tool
AH	Ancestral Haplotype
AIDS	Acquired immunodeficiency syndrome
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ANAPC5	Anaphase promoting complex subunit 5
ANI	Asymptomatic neurocognitive impairment
ART	Antiretroviral therapy
ATP	Adenosine triphosphate
BAT1	HLA-B associated transcript 1 (gene)
BDNF	Brain derived neurotrophic factor
bp	Base pairs
BPNS	Brief Peripheral Neuropathy Screening Tool
CaMKI	Calcium/calmodulin dependent kinase 1
CaMKIV	Calcium/calmodulin dependent kinase 4
CaMKK2	Calcium/calmodulin dependent kinase kinase 2
CCL2	Chemokine CC motif ligand 2
CCR5	Chemokine CC motif receptor 5
CD14	Cluster of differentiation 14
CD3	Cluster of differentiation 3
CD4	Cluster of differentiation 4
CHIRI	Curtin Health Innovation Research Institute
CI	Confidence interval
CNS	Central nervous system
CXCR4	Chemokine CXC motif receptor 4
d4T	Stavudine
DAPI	4’,6-diamidino-2-phylindole, dihydrochloride
DNA	Deoxyribose nucleic acid
DRG	Dorsal root ganglia
EDTA	Ethylenediaminetetraacetic acid
EMLA	Eutectic Mixture of Local Anaesthetics
eQTL	Expression quantitative trait loci
FITC	Fluorescein isothiocyanate
gp120	Glycoprotein 120
HAD	HIV-associated dementia
HAND	HIV-associated neurocognitive disorder
HIV	Human immunodeficiency virus
HIV-SN	HIV-associated sensory neuropathy
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
HWE	Hardy-Weinberg equilibrium
IENFD	Intraepidermal nerve fibre density
IgG	Immunoglobulin G
IL	Interleukin

IL17A	Interleukin 17 receptor A
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
LD	Linkage disequilibrium
MAF	Minor allele frequency
MAPK	Mitogen activated protein kinase
MHC	Major histocompatibility complex
ml	Millilitre
MND	Mild neurocognitive disorder
NC	Nerve conduction study test
NCS	Nerve conduction study test
NFKBIL	Nuclear factor kappa light chain gene in B cells inhibitor like 1
NRTI	Nucleoside reverse transcriptase inhibitor
OR	Odds ratio
P2X4R	Purinergic P2X receptor 4
P2X7R	Purinergic P2X receptor 7
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PGP9.5	Protein gene product 9.5
PLWH	People living with HIV
RANTES	Regulated upon activation, normal T-cell expressed and secreted
RNA	ribonucleic acid
RT	Room Temperature
SIRT1	sirtuin 1
SNP	Single nucleotide polymorphism
SSW	Stimulated skin wrinkling
TB	Tuberculosis
TNF	tumour necrosis factor alpha
TNFR	tumour necrosis factor receptor
UTR	untranslated region
WHO	World Health Organisation
µl	Microliter
µm	Micrometre

Table of Contents

Chapter 1

Introduction

This thesis begins with the hypothesis that many HIV-associated neurological diseases exhibit an inflammatory aetiology, and that disease severity is influenced by variations in genes involved in inflammatory and neurological pathways. This thesis addresses the role of two blocks of genes involved in inflammation and neuronal growth/repair in two common neurological complications of HIV infection:

1) *HIV-associated sensory neuropathy (HIV-SN)*
2) *HIV-associated neurocognitive disorder (HAND)*

Chapter 1 will first describe the epidemiology and clinical features of HIV-SN and HAND. This is followed by an introduction to the plausible roles for the TNF- and P2X-block of genes in HIV-SN and HAND based on genetic and experimental evidence. Finally, I will describe the clinical and scientific relevance, the aims, and the hypotheses of this thesis.

1.0 Introduction

1.1 An introduction to the neurological sequelae of HIV infection

Around 37 million people worldwide are living with HIV, with approximately 7.7 million cases in South Africa (https://cfs.hivci.org/country-factsheet.html) and 640,000 in Indonesia (https://cfs.hivci.org/country-factsheet.html) in 2018. A cure or vaccine for HIV remains elusive and, while antiretroviral therapy (ART) has greatly reduced the severity of HIV disease, neurological complications remain common and severely impair the quality of life of those affected [1, 2].

Neurological complications of HIV infection are common in both untreated and treated individuals, occur at all stages of infection, and affect the central (CNS) and peripheral nervous systems (PNS) [3-7]. The underlying neurotoxicity is attributed to inflammation resulting directly and indirectly from HIV itself and the chronic immune activation characteristic of HIV infection [3-5]. Therefore, it is plausible that common pathogenic mechanisms contribute to all neurological sequelae of HIV infection. Furthermore, disease severity may be influenced by an individual's inflammatory profile during HIV infection and their ability to repair neurons following neuronal insult.

Several comprehensive reviews describe the neurological manifestations of HIV infection [3-9]. However, this thesis will focus on two of the most prevalent conditions; HIV-associated sensory neuropathy (HIV-SN) and HIV-associated neurocognitive disorders (HAND). The pathology, pathogenesis and risk factors of HIV-SN and HAND will now be detailed in sections 1.2 and 1.3.

1.2 HIV-associated sensory neuropathy

1.2.1 Overview of HIV-SN

HIV-SN is one of the most common neurological complications associated with HIV infection [10-13]. Historically, about 30% of treatment-naive patients with advanced HIV disease [11, 12], and up to 60% of patients receiving neurotoxic ART, notably stavudine, experience

sensory neuropathy [10, 13]. HIV-SN is a complex disease influenced by demographic, clinical, and genetic risk factors [13-15].

1.2.2 Clinical and pathological features of HIV-SN

HIV-SN is a distal sensory polyneuropathy which initially affects small fibres and progresses to small and large myelinated fibres causing impaired nociception and thermal perception. Symptoms occur bilaterally and ascend proximally, and may include numbness, 'pins and needles' or pain described as 'aching' or 'burning' in the feet and lower legs [16-18]. Patients may also experience allodynia (pain resulting from non-noxious stimuli), hyperalgesia (reduced pain threshold), absent ankle reflexes and loss of sensation in the feet [16-18]. Pathological features of HIV-SN include neuronal loss in sensory dorsal root ganglia (DRG), degeneration of long axons in a 'die-back' manner, a loss of primary afferent sensory terminals in the epidermis and reduced intraepidermal nerve fibre density (IENFD) [19-21]. There is also an underlying inflammatory pathology with an infiltration of macrophages around DRG, higher density of activated macrophages and increased levels of pro-inflammatory cytokines in peripheral nerves, and increased expression of chemokine receptors on CD14+ and CD3+ cells surrounding intraepidermal nerve fibres in the ankle [7, 22-24].

1.2.3 Pathogenesis of HIV-SN

The mechanisms leading to the development of HIV-SN are not fully resolved. HIV-SN comprises two clinically indistinguishable conditions; HIV-associated distal sensory polyneuropathy (HIV-DSP) and antiretroviral toxic neuropathy (ATN). HIV does not appear to infect neurons but experimental evidence suggest HIV encoded proteins, particularly viral envelope glycoprotein 120 (gp120), may play a direct and indirect role in the initiation of neurotoxic inflammatory responses leading to HIV-DSP [17, 25-29]. Gp120 can interact directly with chemokine receptors expressed on neurons, or indirectly through the receptors expressed by surrounding Schwann cells and macrophages. Direct or indirect activation results in the release of pro-inflammatory cytokines and/or apoptosis. For example, DRG sensory neurons and Schwann cells exposed to gp120 which can act on the C-X-C chemokine receptor type 4 (CXCR4), triggers upregulation of CCL5 which stimulates release of tumour necrosis factor-alpha (TNF) and consequent neuronal apoptosis [28].

Dideoxynucleoside reverse transcriptase inhibitors (NRTI; notably stavudine (d4T)) used to treat HIV infection have been associated with higher rates of HIV-SN [10, 30-32]. While ART-associated inflammation may play a role, the higher rates of HIV-SN in these patients is primarily attributed to NRTI-induced mitochondrial dysfunction [30, 31, 33]. Neurotoxic ART such as stavudine has now been substituted with safer therapies, but the impact this has had on the prevalence and risk factors of HIV-SN is not well documented and is addressed here.

1.2.4 HIV-SN and stavudine

Following the discontinued use of stavudine in 2010, anecdotal evidence suggested that HIV-SN remained a common burden. Our group demonstrated in 2016 that HIV-SN affects around 14% of Indonesians treated with stavudine-free ART, compared to 34% of patients at the same clinic receiving stavudine in 2006 [15, 34, 35]. Furthermore, when treated with stavudine, a patient's age and height associated with HIV-SN, whereas in patients treated with stavudine-free ART, HIV-SN associated with greater than 500 copies of HIV RNA/ml and less than 200 CD4 T-cells/µl [15, 34]. So, HIV-SN remains an important neurological complication of HIV infection in Indonesians, but the prevalence is lower and risk factors are markers of HIV-disease. This establishes the possibility that genetic risk factors may too differ without stavudine. Studies considering other stavudine-free populations and genetic risk factors are lacking. This thesis assesses the prevalence and risk factors in HIV+ Africans treated without stavudine, and genetic risk factors in HIV+ Indonesians and Africans treated without stavudine (Chapters 2-6).

1.2.5 Genetic risk factors of HIV-SN

Genetic studies aim to identify risk markers and pathogenic mechanisms. Given the pathological features of HIV-SN, several studies from our group and other teams have investigated genes involved in pain sensation, immunity and inflammation, and neurological pathways [36-42]. This thesis will focus on two blocks of genes involved in inflammatory, neurotransmission and neuronal growth/repair pathways which our group has previously linked with HIV-SN; the *TNF*-block and the *P2X*-block.

1.2.5.1 The *TNF*-block in HIV-SN

TNF is a potent pro-inflammatory cytokine involved in inflammation and apoptosis. Dysregulated TNF expression is associated with several inflammatory conditions (reviewed in [43]). HIV patients display elevated plasma TNF and TNF soluble receptor levels [44]. Increased levels of serum TNF and plasma TNF receptor has been described in several neuropathies including diabetic neuropathy, multifocal motor neuropathy, chronic inflammatory demyelinating polyneuropathy and Guillan-Barre syndrome [45-49]. Furthermore, TNF expression has been demonstrated histologically in sensory fibre axonal and demyelinating neuropathy samples, particularly in samples from individuals experiencing pain [50]. A role for TNF in HIV-SN is supported by animal studies. Exposure of the sciatic nerve of a rat to gp120 resulted in increased expression of TNF and CXCR4 in the DRG and the lumbar spinal dorsal horn [51]. Furthermore, inhibition of TNF with a TNF-soluble receptor expressed by a non-replicating herpes simplex virus vector encoding the p55TNFSR gene reversed gp120-induced mechanical allodynia.

The gene encoding TNF (*TNF*) is located in the central MHC in a region of defined linkage disequilibrium (LD) termed the *TNF*-block. This block includes several immunoregulatory genes; TNF, LTB, LTA, NCR3, LST1, NFKBIL1, ATP6V1G2, BAT1 and MCCD1. Single nucleotide polymorphisms (SNP) within this region have been associated with altered circulating levels of TNF. However due to LD in the region, it is possible that the associated SNP may not be causative [52]. Carriage of the minor allele of TNF-1031 (rs1799964) associated with increased risk of HIV-SN in Caucasian Australians and Indonesian HIV+ patients treated with stavudine [15, 39, 53] but was not associated with HIV-SN in Africans [54]. Furthermore, a haplotype containing the minor allele of rs1799964 associated with HIV-SN in Indonesians and Caucasians but not Africans, and characterisation of TNF-block haplotypes in multiple ethnicities revealed that this haplotype is not present in Africans [55]. In Africans treated with stavudine, the minor alleles of seven SNP (rs11796*A, rs3130059*G, rs2071594*C, rs2071592*A, rs2071591*A, rs909253*G, and rs1041981*C) associated with lower rates of HIV-SN and one haplotype lacking these minor alleles associated with increased risk of HIV-SN. The data suggest *TNF* genotype may influence susceptibility to HIV-SN in patients of

several ethnicities treated with stavudine. Patients treated without stavudine are investigated here.

1.2.5.2 The *P2X*-block in HIV-SN

The *P2X*-block encodes four proteins, purinergic P2X receptors 7 and 4 (P2X7R and P2X4R), calcium/calmodulin-dependent kinase kinase 2 (CaMKK2) and anaphase promoting complex subunit 5 (AnapC5). These proteins are expressed in the peripheral (PNS) and central nervous systems (CNS) and play critical roles in inflammatory and neurological pathways. P2X7R and P2X4R are adenosine triphosphate (ATP)-gated non-specific cation channels which form homo- and heterotrimers and large membrane pores (permeable to molecules up to 900 Daltons). Several P2X7R and P2X4R isoforms exist with differing levels of expression and function, so SNP that affect splicing may influence disease progression [56].

P2X7R can influence inflammatory responses. Stimulation of P2X7R initiates apoptosis, phagocytosis or production of inflammatory molecules including interleukin-1 beta (IL-1β), IL-6 and TNF [57]. A role for P2X7R in animal models and in human studies of neuropathic pain is well established [56, 58]. For example, IL-1β and P2X7R expression by monocytes and lymphocytes were upregulated in patients with neuropathic pain compared to controls [57]. Furthermore, P2X7R is highly polymorphic and several P2X7R SNP have been associated with susceptibility to inflammatory, neurodegenerative and neuropsychological conditions including multiple sclerosis, tuberculosis, bipolar disorder and Alzheimer's disease [59-61].

P2X4R may also influence inflammation, neurotransmission and pain. Stimulation of P2X4R in macrophages and microglia drives an influx of calcium ions activating the p38 mitogen-activated protein kinase (MAPK) [62] leading to release of neurotrophic factors and cytokines such as brain derived neurotrophic factor (BDNF) and TNF. The secretion of BDNF stimulates Tyrosine receptor kinase B receptors in the dorsal horn resulting in downregulation of potassium chloride co-transporter KCC2 [63, 64]. This increases intracellular chloride and disrupts the transmembrane anionic gradient. The chloride gradient influences gamma-aminobutyric acid (GABA) receptor activity depends on chloride gradient, so reductions of KCC2 alters GABA-dependent inhibition of synaptic transmission and increased excitation of lamina 1 neurons leading to hypersensitivity to pain [63].

CaMKK2 is expressed at high levels within the nervous system and is vital in neuronal growth and repair [65, 66]. CaMKK2 is phosphorylated and activated by upstream kinases and exhibits autonomous activity when bound to calcium/calmodulin. Following activation, CaMKK2 phosphorylates several substrates; calcium/calmodulin kinase 4 and 1 (CAMKIV and CAMKI), AMP-activated protein kinase (AMPK) and sirtuin 1 (SIRT1) [65, 67, 68]. These substrates are involved in axonal elongation, neurite outgrowth, and neuronal survival and repair pathways [69-71] as well as production of inflammatory molecules and spreading of macrophages [65]. CaMKK2 may contribute to peripheral neuropathy and neuropathic pain pathways [64, 72, 73]. One such pathway involves the phosphorylation of CAMKIV. This causes an upregulations cyclic-AMP response element-binding protein and nuclear factor-Kβ, which stimulates production of BDNF encouraging neuronal growth and repair. Dysregulated release of BDNF has been linked with neuropathic pain [64].

AnapC5 is one of 12 or more subunits comprising the anaphase promoting complex (APC/C). The APC/C is a ubiquitin ligase which marks regulatory proteins for degradation by the 26s proteasome thus permitting cell cycle progression. Neuronal replication disrupts signal transduction so re-entry into the cell cycle triggers apoptosis [74, 75]. SNP altering the function or expression of ANAPC5 may contribute to the neuronal loss characteristic of HIV-SN. Furthermore, AnapC5 hampers IL-17 signalling by interacting directly with the receptor IL-17RA. This potentially impairs immune responses to infection and is associated with neuroinflammatory diseases such as multiple sclerosis [76, 77]. Additional relevant pathways are detailed in Chapters 3 and 4.

I have previously demonstrated a link between SNP and altered concentrations of TNF in cells from healthy adults stimulated *in vitro*, supporting role for the *P2X*-block in inflammation (Appendix 2 [78]). Furthermore, our group previously associated SNP and haplotypes within the *P2X*-block with HIV-SN in South Africans treated with stavudine [79]. The minor allele of three SNP in *CAMKK2* (s1560568, rs2686344 and rs2686367) associated with HIV-SN in Southern Africans treated with stavudine and the minor alleles of rs1560568 and rs2686367 remained independently associated with higher rates of HIV-SN after adjusting for patient age and height. Six CaMKK2 haplotypes associated with HIV-SN of which two remained

independently associated after adjusting for patient age height. These studies highlight a link between the P2X-block, most notably with *CAMKK2*.

Previous investigations of the *TNF*- and the *P2X*- block highlight potential roles for the encoded proteins in the pathogenesis of HIV-SN. However, these investigations considered patients receiving stavudine, a known neurotoxic drug associated with increased rates of HIV-SN. We cannot determine if associations with HIV-SN reflect mechanisms/pathways invoked by the use of stavudine. It is now pertinent to determine if these genes contribute to HIV-SN in the post-stavudine era. This thesis explores for the first time the role of the *TNF*-block and the *P2X*-block of genes in HIV-SN in patients treated with stavudine-free ART (addressed in chapters 2-6).

1.3 HIV-associated neurocognitive disorder

1.3.1 Overview of HAND

HIV-associated neurocognitive disorder (HAND) is a primary neurological condition associated with HIV infection within the central nervous system (CNS). HAND encompasses a spectrum of neurological deficits including asymptomatic neurocognitive impairment (ANI), mild neurocognitive disorder (MND), and HIV-associated dementia (HAD) [80]. The advent of effective ART regimens has reduced the severity of neurocognitive impairment and drastically decreased the frequency of frank HAD [5]. However, milder forms of HAND remain common, impacting around 50% of HIV infected individuals even with treatment of ART and optimal viral suppression [81-86]. Unlike HIV-SN, increased rates of HAND have not been associated with stavudine use.

1.3.2 Clinical and pathological features of HAND

Individuals with HAND may experience varying levels of impairment across several or all neurocognitive domains. This may result in difficulties with attention and memory recall, speech deficits, and behavioural changes and motor dysfunction. The severity of impairment differs by neurocognitive domain [3, 81, 84]. Symptoms may impair an individual's capability to work, reflect or reduce adherence to ART, and impact quality of life [2, 87]. Pathology of HAND includes neuronal loss, degeneration of synapses and neuroinflammation. HIV

encephalitis and neuronal loss were common features of HAD [88]. However, inflammation marked by microglial activation and production of cytokines and chemokines, and synaptic degeneration correlate with milder forms of neurocognitive impairment including ANI and MND [82, 89, 90]. HAND can impact HIV+ individuals early after HIV infection. Initiation of ART may prevent severe symptoms of HAND developing or improve existing neurocognitive symptoms. However, ART does not always improve neurocognitive impairments, most notably in the memory domain [91, 92].

1.3.3 Pathogenesis of HAND

Experimental evidence highlights neuroinflammation as a crucial mediator of neurodegeneration observed in HAND [81-86]. HIV enters the CNS soon after infection. One route of entry involves HIV-infected monocytes and CD4+ T-cells crossing the blood brain barrier where the infected cells activate astrocytes and microglia [93-95]. This triggers production of pro-inflammatory cytokines and chemokines including TNF, IL-6, IL-1β and chemokine C-C motif ligand 2 (CCL2), and release of viral proteins including gp120 and transactivator of transcription (Tat). This causes release of ATP and calcium ions, and leads to oxidative stress and further inflammation which is associated with neuronal and synaptic degeneration characteristic of HAND [96, 97]. Identification of the precise mechanisms mediating the neuronal degeneration are hampered by the lack of suitable animal and in vitro models or ex vivo tissue and autopsy material from donors of different stages of HAND. Genetic investigations may help us to identify the underlying mechanisms.

1.3.4 Genetic risk factors of HAND

As with HIV-SN (described in 1.2.5), many genetic studies of HAND focus on genes involved in inflammatory pathways, including genes within the *TNF*-block [98-102]. Equivalent studies of genes which contribute to neuronal growth/repair pathways are lacking. Given the link with the *P2X*-block in HIV-SN in Africans treated with stavudine and with altered expression of TNF, Chapters 7 and 8 will explore associations between SNP in the *P2X*-block and HAND.

1.3.4.1 *P2X*-block in HAND

As described in 1.2.5.2, the P2X-block encodes P2X7R, P2X4R and CaMKK2 which are involved in inflammatory and neurological pathways in the PNS and CNS, and may too contribute to

HAND. P2X7R and P2X4R are involved in astrocyte and microglial activation and production of TNF, IL-1β and IL-6 [62, 103, 104] which are associated with synaptic and neuronal loss. P2X7R is implicated in animal models of HAND. In a rodent model of gp120-induced cognitive impairment, the expression of P2X7R in the hippocampus was markedly higher than in the controls [105].

CaMKK2 is expressed in high concentrations in the brain [65]. As described in 1.2.5.2, CaMKK2 is essential in neuronal growth/repair pathways but also plays a pivotal role in synapse strengthening and long term memory potentiation. Given this role, CaMKK2 has been implicated in Alzheimer's disease, schizophrenia and anxiety disorders [59, 106, 107] and is a plausible candidate in HAND. Accordingly, a *CAMKK2* intronic SNP, rs1063843, was associated with decreased expression of CaMKK2 in the dorsolateral prefrontal cortex, deficits in working memory and executive function, and increased risk of schizophrenia [107]. The effect of the P2X-block genotypes in HAND are investigated in Chapters 7 and 8.

1.4 Clinical Relevance of this thesis

The introduction and availability of ART has significantly reduced the severity of HIV disease, but neurological complications such as HIV-SN and HAND remain common and severely impact an individual's quality of life. The discontinuation of neurotoxic ART, notably stavudine, is a welcome change. Clarification of demographic, clinical and genetic risk factors of HIV-SN without stavudine will more clearly illuminate pathways involved in the pathogenesis of HIV-SN and help clinicians identify, monitor and mitigate patient risk. A targeted investigation including previously associated genes along with neighbouring candidate genes may help elucidate the underlying mechanisms of HIV-SN.

Furthermore, neurocognitive impairment is associated with inflammation and neurodegeneration. Therefore investigation of candidate genes involved in neuronal growth/repair and inflammation and their role in neurocognitive impairment may also identify at-risk patients requiring specialised care and help identify underlying mechanisms. No treatments currently prevent or cure HIV-SN or HAND. Therefore, identification of molecular mechanisms will be of great clinical value.

1.5 Hypothesis and Aims of this thesis

Hypothesis: HIV infection and its treatment lead to inflammation and neuronal degeneration resulting in neurological disease, notably HIV-SN and HAND. The severity and recovery of HIV-SN and HAND, in addition to demographic and clinical factors, is influenced by host inflammatory and neuronal growth/repair genotypes – the *TNF*- and *P2X*-block. Furthermore, effects of genetic risk factors are mediated by altered expression or function of the encoded proteins.

Study Populations: This work was made possible because three cohorts of HIV+ patients treated without stavudine were available in Indonesia and South Africa. The following cohorts will be used to address the Aims of this thesis.

1. Indonesian HIV-SN cohort – Participants were recruited by our colleagues Dr Fitri Octaviana, Dr Yanuar Ahmed Safri and Dr Denise Dewanto at the Cipto Mangunkusumo National Referral Hospital in Jakarta in 2016.
2. South African HIV-SN cohort – Participants were recruited by our colleagues Dr Prinisha Pillay and Dr Huguette Gaelle Ngassa Mbenda at the Lenasia South Community Health Hospital in Johannesburg in 2016.
3. Indonesian HAND cohort – Participants were recruited for the JakCCANDO study [108] by our colleagues Dr Riwanti Estiasari and Dr Dinda Diafiri the Cipto Mangunkusumo National Referral Hospital in Jakarta in 2013. Participants in the JackCCANDO study were previously genotyped for a subset of the *P2X*-block SNP but only two *TNF*-block SNP.

Aim 1: To determine demographic, clinical and the *TNF*- and *P2X*-block genetic markers of HIV-SN in Indonesians and Africans treated without stavudine. Additionally, to determine the prevalence and demographic and clinical risk factors of HIV-SN in Africans treated without stavudine. This study addresses the following questions:

- Has the prevalence and risk factors of HIV-SN changed in African patients treated without stavudine?

- Do *TNF*-block SNP and haplotypes previously associated with HIV-SN remain a marker of risk in South Africans and Indonesians treated without stavudine? (Chapters 2)
- Do previously identified and newly included *P2X*-block SNP and haplotypes associate with HIV-SN in Africans treated without stavudine? Do *P2X*-block SNP associate with HIV-SN in Indonesians treated without stavudine? (Chapters 3 and 4)
- Are associations between *P2X*-block SNP and HIV-SN specific to small or large fibre pathology? (Chapter 5)

Aim 2: To determine if the expression of the proteins encoded by the *P2X*-block in the epidermis of skin from HIV-SN patients differs from that observed in HIV+ patients without HIV-SN and healthy controls. The investigation presented in Chapter 6 provides insight into the proteins involved in HIV-SN by answering the following questions:

- Do individuals with HIV-SN have greater amounts of P2X7R, P2X4R and CaMKK2 in the epidermis compared to patients without HIV-SN or healthy controls?
- Are P2X7R, P2X4R and CaMKK2 co-located with intraepidermal nerve fibres? Does the location of P2X7R, P2X4R and CaMKK2 differ in patients with and without HIV-SN and healthy controls?
- Is the intraepidermal nerve fibre density decreased in patients with HIV-SN compared to patients without HIV-SN and healthy controls?

Aim 3: To determine if *P2X*-block SNP are associated with neurocognitive impairment in HIV+ Indonesians. The investigations presented in Chapters 7 and 8 inform the following questions:

- Do *P2X*-block SNP associate with neurocognitive assessment scores in HIV+ Indonesians as they commence ART and during the first 12 months of receiving ART?
- Do *P2X*-block SNP associate with neurocognitive assessment scores for different neurocognitive domains?

1.6 Introduction References

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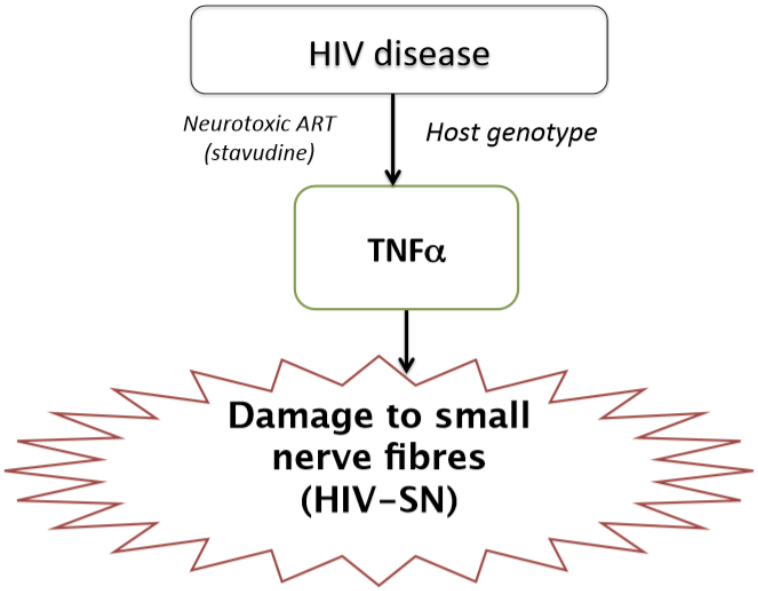
Chapter 2

TNF-Block Genotypes Influence Susceptibility to HIV-Associated Sensory Neuropathy in Indonesians and South Africans

In this chapter, I assessed whether published associations between polymorphisms and haplotypes from the TNF-block and HIV-SN were unique to patients receiving neurotoxic stavudine TNF-block genotypes associated with HIV-SN in two ethnicities were compared to identify critical alleles within conserved haplotypes.



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TNF-Block Genotypes Influence Susceptibility to HIV-Associated Sensory Neuropathy in Indonesians and South Africans

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Abstract: HIV-associated sensory neuropathy (HIV-SN) is a disabling complication of HIV disease and antiretroviral therapies (ART). Since stavudine was removed from recommended treatment schedules, the prevalence of HIV-SN has declined and associated risk factors have changed. With stavudine, rs1799964*C (TNF-1031) associated with HIV-SN in Caucasians and Indonesians but not in South Africans. Here, we investigate associations between HIV-SN and rs1799964*C and 12 other polymorphisms spanning *TNF* and seven neighboring genes (the *TNF*-block) in Indonesians (n = 202; 34/168 cases) and South Africans (n = 75; 29/75 cases) treated without stavudine. Haplotypes were derived using fastPHASE and haplotype networks built with PopART. There were no associations with rs1799964*C in either population. However, rs9281523*C in intron 10 of *BAT1* (alternatively *DDX39B*) independently associated with HIV-SN in Indonesians after correcting for lower CD4 T-cell counts and >500 copies of HIV RNA/mL (model p = 0.0011, Pseudo R^2 = 0.09). rs4947324*T (between *NFKBIL1* and *LTA*) independently associated with reduced risk of HIV-SN and shared haplotype 1 (containing no minor alleles) associated with increased risk of HIV-SN after correcting for greater body weight, a history of tuberculosis and nadir CD4 T-cell counts (model: p = 0.0003, Pseudo R^2 = 0.22). These results confirm *TNF*-block genotypes influence susceptibility of HIV-SN. However, critical genotypes differ between ethnicities and with stavudine use.

Keywords: HIV; sensory neuropathy; *TNF*-block; 8.1 ancestral haplotype; *DDX39B* and *BAT1*

1. Introduction

HIV-associated sensory neuropathy (HIV-SN) is a disabling complication of HIV disease and its treatment. It predominately affects nerve fibers that innervate the distal limbs, particularly the feet [1]. Symptoms include pain, burning and numbness, which impact an individual's quality of life and ability to work [2,3]. Nucleoside reverse transcriptase inhibitors (NRTI) are effective anti-retroviral therapies (ART) but some, most notably stavudine (d4T), have severe adverse effects, including sensory neuropathy and lipodystrophy [4]. The prevalence of HIV-SN varied from 19% to 57% in patients exposed to stavudine [5–9]. Stavudine has not been recommended since 2010 due to its toxicity and a reduction in HIV-SN cases have been noted but not well documented.

We compared the prevalence of HIV-SN assessed with the AIDS Clinical Trial Group Brief Peripheral Neuropathy Screening Tool in HIV+ patients treated at an inner-city clinic in Jakarta, Indonesia with stavudine in 2006 and without stavudine in 2016 [10]. A patient's height and age were associated with HIV-SN among patients receiving stavudine, and 34% experienced neuropathy [5]. In 2016, the prevalence of HIV-SN was 14.2% among patients who had not been exposed to stavudine. Most patients (94%) had <500 copies HIV RNA/mL plasma, but >500 copies HIV RNA/mL and a nadir of below 200 CD4 T-cells/μL associated with HIV-SN. Thus, HIV-SN still presents in patients treated without stavudine, but the prevalence is lower, and the risk factors are markers of HIV disease severity. With the same screening test, the prevalence of HIV-SN was higher (57%) in South African patients receiving stavudine, and was linked with age and height, as in Indonesia [9]. The prevalence of HIV-SN without stavudine in this setting is unclear.

Tumor necrosis factor alpha (TNF) expression has been demonstrated histologically in specimens from individuals with sensory fiber, axonal and/or demyelinating neuropathies, particularly when there was pain [11]. Its role in HIV patients is supported by evidence that the application of HIV gp120 to the sciatic nerve of a rat upregulated expression of TNF, CXCR4 and CXCL12 in the dorsal root ganglia and the lumbar spinal dorsal horn. A non-replicating herpes simplex virus vector encoding the *p55TNFSR* gene (producing a TNF-soluble receptor able to block TNF bioactivity) reversed mechanical allodynia [12]. Since HIV patients display elevated levels of TNF and its soluble receptor in plasma [13], a role in HIV-SN is plausible. However, TNF is difficult to visualize in skin biopsies—perhaps because expression is transient. Genetic associations provide a signature that is stable over time.

The gene encoding TNF (*TNF*) lies in the central MHC in a defined region of high linkage disequilibrium (LD; the *TNF*-block), which contains several potentially pro-inflammatory genes (*TNF*, *LTB*, *LTA*, *NCR3*, *LST1*, *NFKBIL1*, *ATP6V1G2*, *BAT1* (alternatively *DDX39B*) and *MCCD1*) [14]. Carriage of the minor allele at TNF-1031 (rs1799964) associated with increased risk of HIV-SN in Caucasians, Chinese and Malays who had received stavudine [5,6,15], but not in South Africans [16]. We characterized *TNF*-block haplotypes in multiple ethnicities and showed that the haplotype containing the minor allele of TNF-1031 in Asians and Caucasians was not present in Africans [14,16]. This implicates an allele carried in linkage with TNF-1031 in its associations with HIV-SN.

Here, Indonesian and South African HIV patients treated without stavudine are assessed to identify single nucleotide polymorphisms (SNP) and haplotypes of the *TNF*-block associated with HIV-SN. In addition to TNF-1031, we consider markers of the Caucasian 8.1 ancestral haplotype (AH; HLA A*0101: Cw*0701: B*0801: DRB1*0301: DQA1*0501: DQB1*0201), which has been associated with many immunopathological conditions including diabetes and coeliac disease [17]. The most studied candidate polymorphism lies at position -308 upstream of *TNF* (TNF-308; rs1800629). Despite several promising ex vivo studies, alleles of rs1800629 did not affect TNF production when analyzed using promotor constructs [18], so other SNP within the conserved haplotype may be important. As rs1800629*A occurs in several *TNF*-block haplotypes, we use an indel in intron 10 of the *BAT1/DDX39B* gene (rs9281523*C) as a specific marker of the *TNF*-block of the 8.1AH [14]. In Asians, the minor alleles of rs1800629 and rs9281523 occur as part of the diabetogenic 8.1 AH [19,20], consistent with a role for the region in immunopathology. The minor allele of rs9281523 associated with risk of HIV-SN in Caucasian patients who developed a toxic neuropathy following exposure to stavudine [6], but showed no effect in our

cross-sectional studies of Indonesian or South African patients treated with stavudine [5,16]. Here, we assess two cohorts with no exposure to stavudine.

Parallel investigation in two cohorts of different ethnicity has potential to identify critical SNP within conserved haplotypes. Specifically, instances where the predominant haplotypes vary, but the same SNP associates with the phenotype provide circumstantial evidence that the SNP contributes directly to the phenotype.

2. Results

2.1. Measures of the Severity of HIV Disease Predict HIV-SN in Indonesians and Africans

The South African cohort (n = 75) included 29 patients with HIV-SN (38.7%). Participants were relatively young [39 (19–60)] and 60% were female (45/75). Patients with HIV-SN were a little heavier and taller than those without. Height, weight and lower nadir CD4 T-cell counts associated significantly ($p < 0.05$) with HIV-SN. A lower current CD4 T-cell count, a history of tuberculosis and >500 copies of HIV RNA/mL associated weakly ($p < 0.20$) with HIV-SN (Table 1). The regression model retained greater weight, tuberculosis and a low nadir CD4 T-cell count independently associated with HIV-SN (model $p = 0.0007$; pseudo $R^2 = 0.18$; Table S1).

The Indonesian cohort (n = 202) included 34 patients with HIV-SN (16.8%). Participants were relatively young [35 years (19–60 years)] and 29% were female (58/202). A lower current CD4 T-cell count and >500 copies of HIV RNA/mL were significantly associated with HIV-SN in bivariate analyses ($p < 0.05$; Table 1). A lower nadir CD4 T-cell count and a history of tuberculosis were weakly linked with HIV-SN ($p < 0.20$) and were also included multivariate analyses. The regression model identified a current viral load >500 copies of HIV RNA/mL and a lower current CD4 T-cell count as independently associated with HIV-SN ($p = 0.0006$; pseudo $R^2 = 0.08$; Table S1) [21].

We note that height was associated with HIV-SN in African and Indonesian patients treated with stavudine. Here, in Indonesians, height was excluded from logistic regression modeling as it did not meet inclusion criteria ($p = 0.71$; Table 1). However, in Africans, height met criteria for inclusion in logistic regression modelling ($p = 0.03$; Table 1) but was not retained in the optimal model. It is unlikely that associations with body weight in the optimal model in Africans arise through correlations with height (Pearson's $r = 0.225$, $p = 0.06$).

Table 1. HIV-SN associates with CD4 T-cell counts and control of HIV replication.

Variable	Africans			Indonesians		
	HIV-SN			HIV-SN		
	+ve (n = 29)	**−ve (n = 46)**	**p [a,b]**	**+ve (n = 34)**	**−ve (n = 167)**	**p [a,b]**
Age (years)	40 (24–60)	37 (19–58)	0.11	36 (21–59)	35 (19–60)	0.68
Height (cm)	**168 (147–179)**	**163 (135–186) n = 45**	**0.03**	167 (151–185)	167 (142–180) n = 166	0.71
Weight (kg)	**66 (45–112)**	**55 (35–110) n = 44**	**0.03**	59 (39–88)	58.5 (37–104) n = 166	1.00
Current CD4 T-cells/µL	221 (22–685)	300 (8–832)	0.06	**326 (44–729)**	**458 (84–1166)**	**0.003**
Nadir CD4 T-cells/µL	**107 (4–575)**	**223 (8–771)**	**0.002**	54 (3–428)	121 (1–599) n = 165	0.06
HIV RNA >500 copies/mL	21/29 (72%)	25/46 (54%)	0.12	**6/29 (17%)**	**7/163 (4.1%)**	**0.005**
History of Tuberculosis	6/28 (29%)	3/45 (7%)	0.08 [c]	18/35 (53%)	66/168 (39%)	0.09
Female Gender	15/29 (52%)	30/46 (65%)	0.25	9/35 (26%)	49/167 (29%)	0.98

[a] Mann-Whitney test used to assess all continuous variables—Median (range); [b] χ^2 test used to assess dichotomous variables—Proportion (%); [c] Fisher's Exact test used where $n < 5$; Shading marks factors included in logistic regressions. Significant differences are shown in bold font.

2.2. Two Alleles Associated with HIV-SN in Indonesians but not Africans

Thirteen SNP previously linked with HIV-SN either independently or within haplotypes were selected from across the *TNF*-block for genotyping in these cohorts (Table 2) [6,15,16]. In the Indonesian cohort, the minor alleles of rs9281523 (*DDX39B*/*BAT1*(intron10)) and rs1800629 (TNF-308) were more common in patients with HIV-SN. These alleles were in tight LD but the minor allele of rs1800629 also occurred alone, so only rs9281523*C was included in logistic regressions as it reflects carriage of both alleles. No other SNP attained $p < 0.20$—the cut-off for inclusion in logistic regressions. The optimal model included >500 copies HIV RNA/mL, a lower current CD4 T-cell count and the minor allele of rs9281523 ($p = 0.0011$, pseudo $R^2 = 0.09$; Table 3).

Minor alleles of rs2981523 and rs1800629 were present at higher frequencies but were not associated with HIV-SN in Africans (Table 2). However weak associations with reduced risk ($p < 0.20$) were evident with rs4947324*T (between *NFKBIL1* and *LTA*) and rs1041981*C (in *LTA*). These were included in logistic regression modeling. The resulting model ($p = 0.003$, pseudo $R^2 = 0.23$; Table 4) incorporated weight, tuberculosis, lower nadir CD4 T-cell count and rs4947324*T.

Table 2. Alleles of two SNP associate with HIV-SN in Indonesians but not Africans.

SNP rsID	Africans ($n = 75$)					Indonesians ($n = 202$)				
	Minor Allele	MAF [a]	HIV-SN		p [e]	Minor Allele	MAF [a]	HIV-SN		p [d]
			+ve [b]	−ve				+ve [b]	−ve [c]	
rs2075582 (*MCCD1*)	C	0.13	7/28 [d] (25%)	12/46 (27%)	0.87	C	0.34	19/34 [e] (56%)	94/166 (57%)	0.97
rs9281523 (*DDX39B*)	-	0.20	9/29 (31%)	17/46 (37%)	0.60	-	0.03	**5/29 (17%)**	**8/166 (5%)**	**0.03**
rs11796 (*DDX39B*)	A	0.41	16/29 (55%)	31/46 (67%)	0.27	T	0.35	21/35 (60%)	95/167 (57%)	0.73
rs2523506 (*DDX39B*)	T	0.12	6/28 (21%)	9/46 (20%)	0.85	T	0.34	13/34 (38%)	70/165 (42%)	0.65
rs2523504 (*intergenic*)	T	0.17	8/29 (28%)	15/46 (33%)	0.60	T	0.33	19/35 (54%)	92/165 (56%)	0.87
rs2071594 (*intergenic*)	G	0.41	16/29 (55%)	31/46 (67%)	0.29	C	0.37	21/35 (60%)	98/165 (59%)	0.95
rs2071593 (*intergenic*)	A	0.07	2/29 [f] (7%)	8/46 (17%)	0.30	A	0.12	7/34 (21%)	38/167 (23%)	1.00
rs2071592 (*NFKBIL1*)	T	0.42	16/29 (55%)	30/46 (67%)	0.32	A	0.30	19/34 (56%)	83/166 (50%)	0.53
rs4947324 (*intergenic*)	T	0.16	6/29 (21%)	16/46 (35%)	0.19	T	0.03	3/35 (9%)	8/166 (5%)	0.41
rs909253 (*LTA*)	A	0.41	16/29 (55%)	31/46 (67%)	0.29	G	0.36	21/35 (60%)	99/167 (59%)	0.94
rs1041981 (*LTA*)	C	0.39	15/29 (52%)	30/46 (67%)	0.20	A	0.36	21/35 (60%)	99/167 (59%)	0.94
rs1799964 (*intergenic*)	C	0.15	6/29 (21%)	12/46 (27%)	0.56	C	0.27	16/35 (46%)	76/165 (46%)	0.97
rs1800629 (*intergenic*)	A	0.23	10/29 (34%)	19/46 (41%)	0.56	A	0.04	**6/35 (17%)**	**11/167 (7%)**	**0.04**

[a] *MAF*: minor allele frequency; [b] Individuals with HIV-SN who carry one or two copies of the minor allele; [c] Individuals without HIV-SN who carry one or two copies of the minor allele; [d] χ^2 or Fisher's Exact test if $n < 5$; [e] Up to six samples failed to genotype for each SNP; [f] Two SNP in Indonesians which associated with HIV-SN ($p < 0.05$) are in bold; Two SNP in Indonesians and two SNP in Africans which met the criteria for inclusion in logistic regression modelling ($p < 0.20$) are shaded.

Table 3. Logistic regression modelling identified rs9281523*C in Indonesians as an independent marker of susceptibility to HIV-SN.

Variable	Odds Ratio	*p* Value	95% Confidence Interval
SNP Model: n = 193 [a], p = 0.0011, Pseudo R^2 = 0.09			
Current CD4 T-cells/μL	1.00	0.02	1.00–1.00
>500 copies HIV RNA/mL	1.86	0.12	0.85–4.07
rs9281523*C	2.49	0.15	0.71–8.65
Haplotype Model: No haplotypes independently associated with HIV-SN after correction for current CD4 T-cells/μL and >500 copies of HIV RNA/mL			

[a] Excluding samples with missing demographic, clinical and/or genotype data.

Table 4. Logistic regression modelling identified rs4947324*T in Africans as an independent marker of susceptibility to HIV-SN.

Variable	Odds Ratio	*p* Value	95% Confidence Interval
SNP Model: n = 71 [a], p = 0.0003, Pseudo R^2 = 0.23			
Weight (kg)	1.04	0.04	1.00–1.08
History of Tuberculosis	5.66	0.04	1.09–29.36
Nadir CD4 T-cells/μL	0.99	0.02	0.99–1.00
rs4947324*T	0.25	0.05	0.06–1.01
Haplotype Model: n = 71 [a], p = 0.0003, Pseudo R^2 = 0.22			
Weight (kg)	1.04	0.02	1.01–1.08
History of Tuberculosis	5.22	0.04	1.09–24.86
Nadir CD4 T-cells/μL	0.99	0.02	0.99–1.00
S1 (Shared Haplotype 1)	3.21	0.07	0.93–11.12

[a] Excluding samples with missing demographic, clinical, genotype data and/or haplotypes perfectly aligned with the absence of HIV-SN.

*2.3. One Haplotype Containing rs9281523*C and rs1800629*A Associated with HIV-SN in Indonesians but Not Africans*

FastPHASE generated 10 haplotypes in Indonesians and 12 haplotypes in Africans present at >1.0%. These accounted for 98% and 96% of each population, respectively (Tables 5 and 6). Eight haplotypes were shared between the two populations (S1–S8) and are numbered in order of the frequency of each haplotype in Africans. An additional four haplotypes were unique to Africans (A1–A4) and two were unique to Indonesians (I1, I2).

The haplotype S2 contained the minor alleles of rs9281523 and rs1800629, and associated with HIV-SN in bivariate analyses of the Indonesian cohort (Table 5) but did not remain in the final model (Table 3). This haplotype was also common in Africans but was not linked with HIV-SN. Three other haplotypes (S1, A3 and S7) carried by Africans met the criterion for inclusion in logistic regression models ($p < 0.20$; Table 6). However, A3 and S7 perfectly predicted the absence of HIV-SN and could not be included. The resulting model ($p = 0.0003$, pseudo $R^2 = 0.22$; Table 4) included greater body weight, prior TB, lower nadir CD4 T-cell counts and S1. S1 contains no minor alleles.

Table 5. One haplotype associated with HIV-SN in Indonesians.

Haplotype [a]	rs2075582	rs9281523	rs11796	rs2523506	rs2523504	rs2071594	rs2071593	rs2071592	rs4947324	rs909253	rs1041981	rs1799964	rs1800629	HIV-SN [b] +ve (*n* = 35)		HIV-SN [b] −ve (*n* = 167)		p^c
S1	T	-	T	G	C	C	G	A	C	G	A	T	G	15	43%	72	43%	0.69
S3	T	-	A	T	C	G	G	T	C	A	C	C	G	14	40%	70	42%	0.85
S4	C	-	A	G	T	G	G	T	C	A	C	T	G	12	34%	60	36%	0.87
S8	C	-	A	G	T	G	A	T	C	A	C	T	G	7	20%	36	22%	0.97
I1	T	-	T	G	C	C	G	T	C	G	A	T	G	3	9%	21	13%	0.77
S6	T	-	A	G	C	G	G	T	C	A	C	T	G	2	6%	10	6%	0.99
S2 [d]	T	C	T	G	C	C	G	A	C	G	A	T	A	**5**	**14%**	**8**	**5%**	**0.02**
S7	T	-	A	G	C	G	G	T	T	A	C	C	G	2	6%	8	5%	0.66
I2	C	-	A	G	C	C	G	A	C	G	A	T	G	0	0%	5	3%	0.99
S5	T	-	T	G	C	C	G	A	C	G	A	T	A	1	3%	3	2%	0.51

[a] Haplotypes shared between Africans and Indonesians are labelled S1–S8 in order of their population frequencies in Africans. Haplotypes unique to Indonesians are labelled I1 and I2 in order of population frequencies. Haplotypes carried at <1% are excluded. Minor alleles for each population are shaded grey; [b] Number of individuals who carry one or two copies of each haplotype; [c] χ^2 (or Fisher's Exact test if $n < 5$); [d] Haplotypes meeting logistic regression criteria ($p < 0.20$) are in bold.

Table 6. Three haplotypes were weakly associated with HIV-SN in Africans.

Haplotype [a]	rs2075582	rs9281523	rs11796	rs2523506	rs2523504	rs2071594	rs2071593	rs2071592	rs4947324	rs909253	rs1041981	rs1799964	rs1800629	HIV-SN [b] +ve (*n* = 29)		HIV-SN [b] −ve (*n* = 46)		p^c
S1 [d]	T	-	T	G	C	C	G	A	C	G	A	T	G	**19**	**66%**	**23**	**50%**	**0.19**
S2	T	C	T	G	C	C	G	A	C	G	A	T	A	9	31%	16	35%	0.34
S3	T	-	A	T	C	G	G	T	C	A	C	C	G	5	17%	8	17%	0.99
A1	T	-	A	G	C	G	G	T	T	A	C	T	G	3	10%	8	17%	0.51
S4	C	-	A	G	T	G	G	T	C	A	C	T	G	3	10%	5	11%	0.99
S5	T	-	T	G	C	C	G	A	C	G	A	T	A	1	3%	4	9%	0.64
A2	C	-	A	G	T	G	A	T	T	A	C	T	G	1	3%	4	9%	0.64
S6	T	-	A	G	C	G	G	T	C	A	C	T	G	3	10%	2	4%	0.37
A3	T	-	T	G	T	C	G	A	C	G	A	T	G	**0**	**0%**	**4**	**9%**	**0.15**
S7	T	-	A	G	C	G	G	T	T	A	C	C	G	**0**	**0%**	**4**	**9%**	**0.15**
S8	C	-	A	G	T	G	A	T	C	A	C	T	G	0	0%	2	4%	0.52
A4	C	-	A	G	T	G	G	T	C	A	A	T	G	0	0%	2	4%	0.52

[a] Haplotypes shared between Africans and Indonesians are labelled S1–S8 in order of their population frequencies in Africans. Haplotypes unique to Africans are labelled A1–A4 in order of population frequencies. Haplotypes carried at <1% are excluded. Minor alleles for each population are shaded grey; [b] Number of individuals who carry one or two copies of each haplotype; [c] χ^2 (or Fisher's Exact test if $n < 5$); [d] Haplotypes meeting logistic regression criteria ($p < 0.20$) are in bold.

2.4. One Haplogroup Contained the Two Haplotypes Associated with HIV-SN in Africans and Indonesians

A haplotype network was constructed for the 14 haplotypes occurring at >1% in Africans or Indonesians; S1–S8, A1–A4 and I1–I2. The haplotype network identified two haplogroups (A and B), where each contained two shared haplotypes and one unique to Africans (Figure 1).

Haplogroup A included S1, S2 and A3—carried by 91% of the Africans and 50% of Indonesians. S1 associated with HIV-SN in Africans and S2 with HIV-SN in Indonesians (Tables 5 and 6). S1 and S2 differ only at rs9281523 (DDX39B/BAT1 (intron10)) and rs1800629 (TNF-308), marking the 8.1AH. A3 perfectly predicted the absence of HIV-SN in Africans.

Haplogroup B contained S4, S8 and A2, and was carried by 20% of Africans and 57% of Indonesians. S8 was also not observed in Africans with HIV-SN. These three haplotypes share seven minor alleles and differ at a further two loci: S8 and A2 include the minor allele of rs2071593 and A2 carries the minor allele of rs4947324, which are associated with reduced risk of HIV-SN in Africans (Table 2).

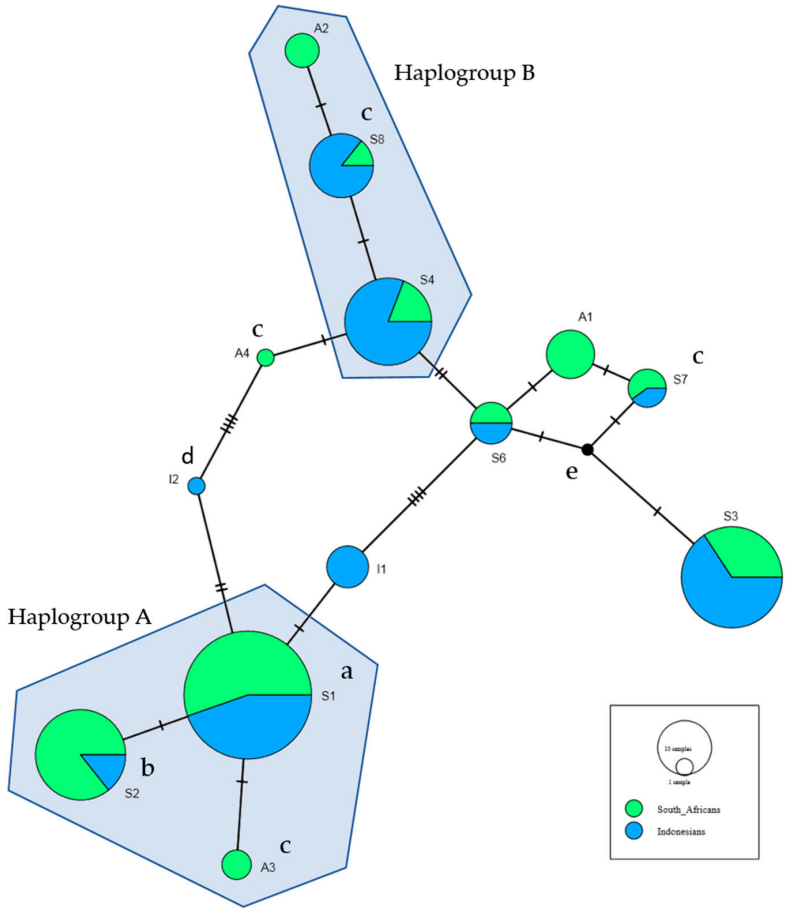

Figure 1. The haplotype network was constructed using all haplotypes which occurred in Africans and Indonesians at greater than 1%. This includes the eight shared haplotypes, four haplotypes unique to Africans and two haplotypes unique to Indonesians, as defined as in Tables 5 and 6. [a] Associated with increased risk of HIV-SN in Africans in bivariate analyses (Table 6). [b] Associated with increased risk of HIV-SN in Indonesians in bivariate analyses (Table 5). [c] Haplotypes not found in Africans with HIV-SN. [d] Haplotypes not found in Indonesians with HIV-SN. [e] Median vector: hypothetical haplotype automatically generated for maximum parsimony.

3. Discussion

Despite the discontinuation of stavudine, HIV-SN remains a common neurological complication of HIV disease, impacting 14% of Indonesians surveyed in the 2016 cross-sectional study [10] and 38% of Africans in this study. We describe associations between HIV-SN and demographic variables, clinical variables and SNP and haplotypes in the *TNF*-block which have previously been linked with HIV-SN in Caucasians, Asians and Africans.

Age and height were strong predictors of HIV-SN in Africans and Indonesians treated with stavudine [5,9]. However, without stavudine, markers of HIV disease severity were more clearly associated with HIV-SN. In African patients, the optimal markers were lower nadir CD4 T-cell counts, greater weight and a history of tuberculosis whilst low current CD4 T-cell counts and >500 HIV RNA copies/mL were the clearest associations with HIV-SN in Indonesians. As the African patients had received ART for only 6–8 months, current CD4 T-cell levels may not have stabilized and a clearer association with nadir CD4 T-cell counts is reasonable. In Africans, tuberculosis remained independently associated with HIV-SN in all regression models (Table S1 and Table 4), despite being present in 41% of Indonesians (*cf* 15% of Africans) and associated weakly with HIV-SN in both populations in bivariate analyses (Table 1). Pyridoxine is routinely provided to patients treated for tuberculosis in South Africa and Indonesia, but a previous South African study linked inadequate

levels of pyridoxine with neuropathy despite administration of supplements [22]. Overall, biological markers of HIV disease were the clearest demographic and clinical associations of HIV-SN. This is consistent with findings prior to the advent of ART (and hence, stavudine) [23] and suggests the underlying mechanisms of HIV-SN may differ between patients treated with and without stavudine.

Differences in the pathogenic pathways are also evidenced by the different genetic associations identified in this study. We found no associations between the minor allele of rs1799964 (TNF-1031) and HIV-SN in Africans or Indonesians evident in our studies of Asians and Caucasians treated with stavudine [5,6,15]. In Indonesians treated without stavudine, rs9281523*C (*DDX39B/BAT1* (intron10)) is independently associated with HIV-SN after correcting for lower current CD4 T-cell counts and >500 copies of HIV RNA/mL (model $p = 0.0011$, Pseudo $R^2 = 0.09$; Table 3). The S2 haplotype, which contains rs9281523*C, was associated with HIV-SN in Indonesians in bivariate analyses. rs9281523*C marks the 8.1AH which has been linked with accelerated loss of CD4 T-cells and impaired recovery following HIV infection [24,25] and with numerous immunopathological diseases [17,26]. So, a link with HIV-SN is plausible and the search for the SNP responsible has wide ramifications. A direct role for rs9281523 is supported by its inclusion in the optimal model for Indonesians, while the associated haplotype (S2) was excluded. However, S2 and S1 (the haplotype linked with risk of HIV-SN in Africans) differed only at rs9281523 and rs1800629 and occurred in the same haplogroup (Figure 1). This suggests the haplotypes may have descended from a common ancestor, but argues against a direct role for rs9281523 or rs1800629. It also fits with the observation that rs9281523 shows no associations with HIV-SN in Africans.

In Africans, the minor alleles of rs4947324*T and rs1041981*C associated weakly with reduced risk of HIV-SN in bivariate analyses (Table 2). Both SNP have been associated with reduced risk of HIV-SN in South Africans treated with stavudine where rs1041981*C and six additional SNP from the *TNF*-block were included in the logistic regression model with age and height [16]. Without stavudine, rs4947324*T alone remained in the optimal model ($p = 0.0003$, Table 4). Three haplotypes (S1, A3 and S7) were weakly associated with altered risk of HIV-SN ($p < 0.20$; Table 6). A3 and S7 were rare haplotypes with no minor alleles in common, but S7 contained rs4947324*T and rs1041981*C. Neither A3 nor S7 occurred in patients with HIV-SN so they could not be included in logistic regression modeling. S1, was independently associated with risk of HIV-SN in the final model along with greater weight, tuberculosis and nadir CD4 T-cells/μL ($p = 0.0003$, Pseudo $R^2 = 0.22$; Table 4). S1 included no minor alleles and therefore contained the major (*C) risk allele of rs4947324.

The search for SNP responsible for associations with *TNF*-block haplotypes is complicated by the lack of relevant in vitro assays for chronic conditions. For example, haplotypes containing polymorphisms within the *TNF*-block were associated with altered TNF and lymphotoxin-alpha production in cultured mononuclear cells from Australian Caucasians but haplotypes containing rs9281523*C and rs4947324*T had no impact [27]. SNP within the *TNF*-block may also impact other MHC genes. The GTEX eQTL database reports altered expression of several immune-related genes within the MHC is associated with carriage of rs9281523*C or rs4947324*T including *C4A*, *HLA-C* and *MICB* (https://gtexportal.org/). However, understanding the biological consequences of these SNP and associated haplotypes is complicated by LD within the region [28].

We recognize the small size of our cohorts, but strict inclusion criteria avoided alternative causes of neuropathy. Further studies are required to validate our findings in larger cohorts when they become available, and to address the biological implications. Overall, we confirm that HIV-SN remains a clinically relevant problem in HIV+ patients treated without stavudine and is more common in those with African ancestry. We confirm that *TNF*-block genotypes associate with HIV-SN but show that different mechanisms are invoked with and without stavudine. Critical genotypes differ between Indonesians and Africans so the causative alleles remain unknown. However, the haplotypes associated with HIV-SN may descend from a common ancestor, and therefore, include allele/s not typed in our panel. Associations with *TNF*-block haplotypes per se confirm the inflammatory etiology of HIV-SN. Moreover, the clearer associations with HIV-SN seen in Africans (evidenced by stronger

regression models) may plausibly align with the greater frequency of HIV-SN in Africans compared with Indonesians. This distinction warrants further investigation.

4. Materials and Methods

4.1. Participants and Phenotypes

HIV-positive adults (n = 185) who had used ART for at least 12 months with no exposure to stavudine were screened for neuropathy at POKDISUS HIV Care Clinic, Cipto Mangunkusumo Hospital, Jakarta, Indonesia in 2016 [21,29]. Patients with any history of other conditions potentially linked with neuropathy were excluded. DNA was also available from nine HIV+ patients with HIV-SN and eight patients without HIV-SN matched by age, gender and CD4 T-cell count. These 17 individuals were recruited in 2012 at the same clinic and met inclusion criteria for the present study. Patients with African ancestry attending the Lenasia South Community Health Hospital, Johannesburg, South Africa, were enrolled after 6–8 months on ART. These studies were approved by the Ethics Committee of the Faculty of Medicine, Universitas Indonesia (approval number: (579/UN2.F1/ETIK/2014), and the Human Research Ethics Committee (Medical) of the University of the Witwatersrand, Johannesburg (approval number: MR121018-R14/49). Written informed consent was obtained in all cases.

HIV-SN was assessed using the AIDS Clinical Trials Group Brief Peripheral Neuropathy Screening Tool, a validated metric for diagnosing HIV-SN [30]. HIV-SN was defined by bilateral presence of at least one symptom (pain/burning/aching, numbness, and paresthesia) and one clinical sign (absent reflexes and impaired vibration sense in the great toe). In South Africans, pinprick sensitivity was added to the BPNS as it provides an assessment specific to small fiber pathology and has high specificity for HIV-SN [31,32]. Clinical and demographic records were collected from medical files to determine risk factors that may be linked with HIV-SN. This included the patient's history of tuberculosis as medications used in its treatment, notably isoniazid, competitively interferes with pyridoxine metabolism and may result in peripheral neuropathy [22,33].

4.2. Genotyping

As described previously [21], genomic DNA was extracted from EDTA-blood samples using Favorprep Blood Genomic DNA Extraction Mini Kits (Favorgen Biotech Corporation, Changzhi, Taiwan) adjusted to 50 ng/µL and diluted 1:1 in TaqMan® OpenArray™ Genotyping Master Mix (Life Technologies, Grand Island, NY, USA). Samples were genotyped for 13 SNP (Table 2) spanning *MMCD1*, *DDX39B*, *ATP6V1G2*, *NFKBIL1*, *LTA* and *TNF* using the QuantStudio 12K Flex Real-Time PCR System on custom TaqMan® OpenArrayTM Real-Time PCR Plates. All alleles were in Hardy-Weinberg Equilibrium (HWE).

4.3. Haplotype Analyses

Haplotypes and their estimated frequencies were determined using the default parameters of the fastPHASE algorithm [34], with haplotypes sampled from the observed genotypes an additional 5000 times per sample. Haplotypes with an estimated frequency less than 1% were excluded from analyses. Haplotypes shared between Africans and Indonesians are labelled S1–S8 in order of their population frequencies in Africans. Haplotypes unique to Africans are labelled A1–A4 and haplotypes unique to Indonesians are labelled I1 and I2 in order of their respective population frequencies. PopART version 1.7 (Population Analysis with Reticulate Trees, Otago, New Zealand; http://popart.otago.ac.nz) was used to construct haplogroups using Median-Joining methods [35].

4.4. Statistical Analyses

Bivariate associations between HIV-SN and demographic or clinical variables, SNP and haplotypes were assessed with t-tests, Mann-Whitney tests, Chi^2 or Fisher's exact tests using GraphPad Prism version 8.2.1 for Windows (Graphpad Software, La Jolla, CA, USA). No corrections were made for

multiple comparisons. All variables which weakly ($p = 0.05–0.20$) or significantly ($p < 0.05$) associated with HIV-SN in bivariate analyses were included in logistic regression modelling. Optimal models identifying variables independently associated with HIV-SN were determined with a stepwise removal process using Stata/IC 16.0 for Windows (StataCorp LLC, College Station, TX, USA).

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/21/2/380/s1.

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Abbreviations

HIV	Human immunodeficiency virus
HIV-SN	HIV-associated sensory neuropathy
BPNS	Brief peripheral neuropathy screening tool
TNF	Tumor necrosis factor
CXCR4	C-X-C chemokine receptor type 4
CXCL12	C-X-C chemokine ligand 12
MHC	Major histocompatibility complex
LD	Linkage disequilibrium
LTB	Lymphotoxin beta
LTA	Lymphotoxin alpha
NCR3	Natural Cytotoxicity Triggering Receptor 3
LST1	Leukocyte-specific transcript 1
NFKBIL1	Nuclear Factor if Kappa Light Polypeptide Gene Enhancer In B-Cells Inhibitor-Like 1
DDX39B	DExD-Box Helicase 39B
MCCD1	Mitochondrial Coiled-Coil Domain 1
AH	Ancestral Haplotype
S1-8	Shared haplotypes 1-8
A1-4	African haplotypes 1-4
I1-2	Indonesian haplotypes 1 and 2
GTEX	Genotype-Tissue Expression
eQTL	Expression quantitative trait loci
C4A	Complement Component 4A
HLA	Human leukocyte antigen
MICB	MHC class I polypeptide-related sequence

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Table S1. Logistic regression modelling identifies demographic and clinical variables independently associating with HIV-SN in Africans and Indonesians.

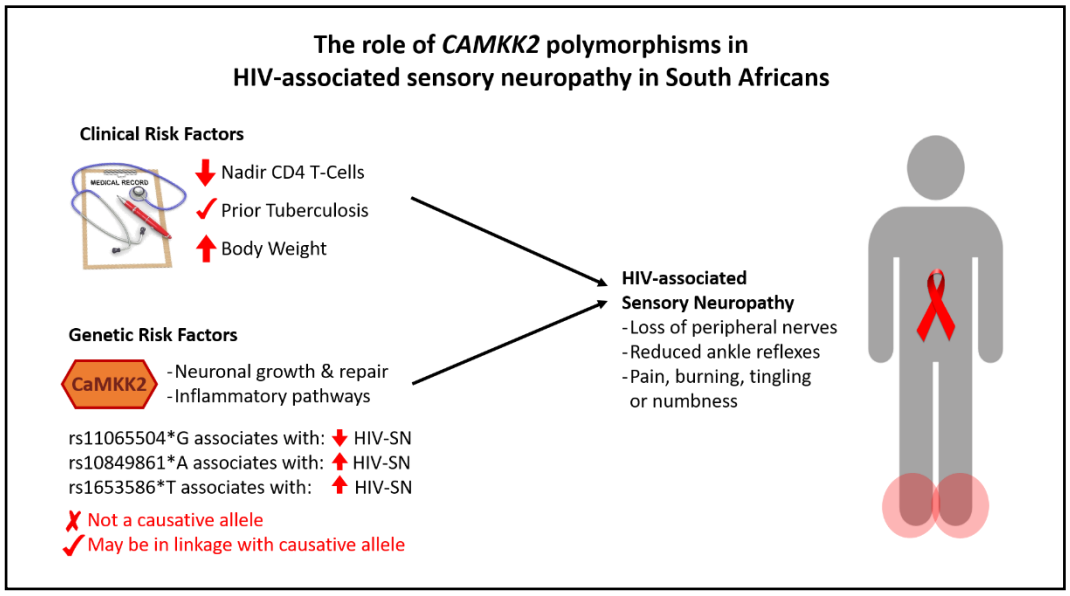
Variable	Odds Ratio	P Value	95% CI
African Optimal Model: n = 71[a], p = 0.0007, Pseudo R^2 = 0.18			
Weight (kg)	1.04	0.03	1.00–1.08
History of Tuberculosis	4.26	0.07	0.90–20.03
Nadir CD4 T-cells/μL	1.00	0.03	0.99–1.00
Indonesian Optimal Model: n = 195[a], p = 0.0006, Pseudo R^2 = 0.08			
Current CD4 T-cells/μL	1.00	0.01	0.99–1.00
>500 copies HIV RNA/mL	3.80	0.04	1.10–13.15

[a] excluding samples with missing demographic, clinical and/or genotype data.

Chapter 3

The role of *CAMKK2* polymorphisms in HIV-Associated Sensory Neuropathy in South Africans

In addition to associations with the TNF-block, polymorphisms and haplotypes of the P2X-block have been associated with HIV-SN in South Africans treated with stavudine. Here, I sought associations in an independent cohort of South African patients treated without stavudine. The data confirm the role of these genes in HIV-SN.



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This chapter was published after Chapter four due to delays in the availability of the South African cohort. Therefore, this chapter refers to results included in Chapter 4.

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The role of *CAMKK2* polymorphisms in HIV-associated sensory neuropathy in South Africans

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Haplotypes

ABSTRACT

Human immunodeficiency virus-associated sensory neuropathy (HIV-SN) is a common neurological complication of HIV infection. It affected 57% of South African patients whose antiretroviral therapy (ART) included stavudine and was influenced by genotypes of the *P2X*-block (*P2X7R*, *P2X4R* and *CAMKK2*). We investigate associations between HIV-SN and *P2X*-block genotypes in patients who never received stavudine. An adjacent gene, *ANAPC5,* was included.

75 HIV+ individuals were assessed using the Brief Peripheral Neuropathy Screen before treatment and after 6-8 months on stavudine-free regimens. DNA was genotyped for 48 polymorphisms across the four genes using an OpenArray™ platform. Haplotypes were derived using fastPHASE. Associations with HIV-SN were assessed using bivariate and multivariate analyses.

Nine individuals (12%) were diagnosed with HIV-SN prior to ART and a further 20 individuals (27%) developed HIV-SN within 6-8 months. Five polymorphisms, rs503720*G (OR = 133) in *P2X7R*, rs10849861*A (OR = 5.99), rs1653586*T (OR = 67.8) and rs11065504*C (OR = 0.02) in *CAMKK2*, and rs2089886*A (OR = 6.68) in *ANAPC5*, associated with HIV-SN after adjusting for body weight, nadir CD4 T-cell counts and prior tuberculosis (model $p < 0.0001$, n = 69, Pseudo $R^2 = 0.54$). Three *CAMKK2* haplotypes were associated with HIV-SN (OR = 2.82, 3.42 and 6.85) after adjusting for body weight, nadir CD4 T-cell counts and prior tuberculosis (model $p < 0.0005$, n = 71, Pseudo $R^2 = 0.26$).

The results support a role for *CAMKK2* in HIV-SN, independent of mechanisms invoked by stavudine.

Significance statement: HIV-associated sensory neuropathy (HIV-SN) remains a clinically relevant complication of HIV infection and its treatment, affecting 38% of patients treated without neurotoxic stavudine. HIV-SN can impact an individual's ability to work and quality of life, with few effective therapeutic options, so an understanding of the underlying mechanisms would have clinical value. We confirm that *CAMKK2* polymorphisms and haplotypes influence susceptibility to HIV-SN in South Africans treated without stavudine. This provides further evidence for a role for the protein encoded by *CAMKK2* in the pathogenesis of HIV-SN, independent of mechanisms initiated by stavudine.

1. Introduction

Human immunodeficiency virus-associated sensory neuropathy (HIV-SN) is a debilitating neurological complication of HIV infection and antiretroviral therapy (ART) [1–4]. The prevalence of HIV-SN in South African patients treated with the nucleoside reverse transcriptase inhibitor (NRTI) stavudine was 57% compared to 38% in those treated with alternative NRTIs, notably Tenofovir [4,5]. HIV-SN can affect an individual's ability to work and quality of life, and existing treatments lack efficacy for managing pain associated with HIV-SN [6], so an understanding of the underlying mechanisms will have clinical value.

An inflammatory pathology of HIV-SN is supported by the observed infiltration of macrophages in dorsal root sensory ganglia, increased density of activated macrophages and production of pro-inflammatory

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cytokines in peripheral nerves, and increased expression of chemokine receptors by $CD14^+$ and $CD3^+$ cells surrounding subcutaneous nerves in the lower leg [7–10]. Furthermore, polymorphisms and haplotypes of *TNF* (and neighbouring genes) are associated with susceptibility to HIV-SN [5,11–13]. P2X7R, P2X4R and CaMKK2 may play a role in this inflammatory pathology.

P2X7R and P2X4R (purinergic receptors 7 and 4) are involved in inflammatory pathways and excitatory neurotransmission following activation by ATP [14]. Stimulation of P2X7R results in interleukin-1β (IL-1β)-dependent induction of tumour necrosis factor (TNF) [15], and the activation of P2X4R causes an influx of calcium ions in microglia. This activates p38 mitogen activated protein kinase (p38-MAPK) resulting in release of brain-derived neurotrophic factor (BDNF) [16] and pro-inflammatory cytokines including TNF [17]. IL1β and TNF can cause hyperexcitability in dorsal neurons causing neuropathic pain [15] and have been implicated in the development of HIV-SN [10,15,18].

CaMKK2 (calcium/calmodulin kinase kinase 2) also induces production of IL-1β and TNF via the p38-MAPK pathway. However, CaMKK2 is expressed in the nervous system and is crucial in neuronal growth and repair [19,20]. CaMKK2 phosphorylates and activates AMPK (AMP-activated protein kinase), SIRT1 (sirtuin 1), CaMKIV (calcium/calmodulin kinase 4) and CaMKI (calcium/calmodulin kinase 1) [19,21,22]. AMPK is associated with neurite outgrowth [19], and SIRT1, CaMKIV and CaMKI are involved in neuronal growth, survival and repair pathways [23–25]. CaMKK2 pathways have been linked to peripheral neuropathy and neuropathic pain [16,26,27]. For example, phosphorylation of CaMKIV upregulates nuclear factor-Kβ and cyclic-AMP response element-binding protein, which triggers release of BDNF and promotes neuron growth and survival. Dysregulated release of BDNF has been linked with neuropathic pain [16].

The encoding genes, *P2X7R*, *P2X4R* and *CAMKK2* (the "*P2X*-block"), lie in a region of linkage disequilibrium (LD) in chromosome 12. We associated alleles of single nucleotide polymorphisms (SNP) in *P2X4R* and *CAMKK2* with concentrations of tumour necrosis factor *in vitro* [28], and with HIV-SN in HIV+ South Africans treated with stavudine [29]. Three co-inherited SNP in *CAMKK2* associated with lower rates of HIV-SN in HIV+ Indonesians treated without stavudine [30].

ANAPC5 lies upstream of *CAMKK2* and exhibits LD with SNP in *CAMKK2*. *ANAPC5* encodes the anaphase promoting complex 5 (AnapC5), involved in progression from metaphase into anaphase. Replication of neurons would disrupt signal transduction so re-entry into the cell cycle triggers apoptosis [31,32]. Hence altered function or expression of *ANAPC5* may promote neuronal death characteristic in HIV-SN. Furthermore, AnapC5 hampers interleukin 17 (IL-17) signalling by interacting directly with the receptor IL-17RA. Reduced IL-17 signalling impairs defence against infections and modifies neuroinflammatory diseases such as multiple sclerosis [33,34].

Here we investigate whether alleles of the *P2X*-block or *ANAPC5* mark susceptibility to HIV-SN inHIV+ South Africans who have never received stavudine.

2. Materials and methods

2.1. Participants and phenotypes

75 HIV+ adults with self-declared African ancestry were enrolled as they commenced first-line ART (usually tenofovir, emtricitabine and efavirenz) at the Lenasia South Community Health Hospital, Johannesburg, South Africa in 2016. Participants were re-assessed 6-8 months after initiating ART. We excluded patients with a clinical history of an illness [other than HIV or tuberculosis (TB)] potentially linked with neuropathy or preventing clinical assessment. Written informed consent was received from all participants and the study was approved by the Human Research Ethics Committee (Medical) of the University of the Witwatersrand, Johannesburg (approval number: MR121018-R14/49).

Participants were assessed for HIV-SN using the Brief Peripheral Neuropathy Screen (BPNS) amended by inclusion of pinprick sensitivity, as it assesses small fibre pathology and has high specificity for HIV-SN [3,35]. Demographic and clinical data were collected from medical files. Data included the participant's age, height, body weight, nadir and current CD4 T-cell count, plasma HIV RNA, and a history of TB as the disease and its treatments may result in peripheral neuropathy [3,36].

2.2. Genotyping and SNP selection

48 SNP from the *P2X*-block and *ANAPC5* were genotyped. 22 SNP within the *P2X*-block were associated with HIV-SN in South Africans treated with stavudine [29]. An additional 16 SNP from the *P2X*-block and 10 SNP in *ANAPC5* were selected based on published links with inflammatory or neurological conditions, and a minimum global minor allele frequency of 5% (https://www.ncbi.nlm.nih.gov/variation/view/).

Genomic DNA was extracted from venous whole blood samples in EDTA using Favorprep Blood Genomic DNA Extraction Mini Kits (Favorgen Biotech Corporation, Changzhi, Taiwan), adjusted to 50ng/μl and diluted 1:1 in TaqMan® OpenArray™ Genotyping Master Mix [5,30]. DNA samples were genotyped using the QuantStudio 12K Flex Real-Time PCR System (Life Technologies, Grand Island, NY, USA) on custom TaqMan® OpenArray™ Real-Time PCR Plates. Genotypes were assigned manually using the OpenArray™ SNP Genotyping Analysis software. Two polymorphisms were monoallelic and all remaining alleles were in Hardy-Weinberg Equilibrium (HWE) in this population.

2.3. Haplotype analyses

Haplotypes and corresponding estimated frequencies were determined using the default parameters of fastPHASE [37], with haplotypes sampled an additional 5,000 times per sample from the observed genotypes. Haplotypes observed in at least two individuals were assessed for associations with HIV-SN. Haplotype networks were constructed using default Median-Joining methods with an epsilon value of 0 in PopART v1.7 (Population Analysis with Reticulate Trees, Otago, New Zealand; http://popart.otago.ac.nz; [38]).

2.4. Statistical analyses

Individuals diagnosed pre-ART (n = 9) and post-ART (n = 20) were grouped to create a cross-sectional study and allow analyses of infrequent demographic and genetic variables. Bivariate associations between HIV-SN and clinical variables, SNP and haplotypes were assessed using T-tests, Mann-Whitney tests, Chi-squared or Fisher's exact tests in GraphPad Prism version 8.2.1 for Windows (Graphpad Software, La Jolla, CA, USA) without corrections for multiple comparisons. Multivariable analyses were then undertaken using Stata/IC 16.0 for Windows (StataCorp LLC, College Station, TX, USA). Logistic regression modelling included clinical and genetic variables with a p-value less than 0.20 on bivariate testing, and a stepwise removal process was used to obtain models of best fit. Logistic regression models excluded samples with missing clinical and genetic information and excluded genetic variables aligned perfectly with risk or protection of HIV-SN.

3. Results

3.1. Clinical factors associated with HIV-SN

We assessed 75 individuals commencing ART and after 6-8 months. All participants received tenofovir, emtricitabine and efavirenz, with the exception of three individuals without HIV-SN who were treated with abacavir, efavirenz and lamivudine. The median age was 39 (19-

Table 1
One SNP in *CAMKK2* associated with reduced risk of HIV-SN.

RSID	Chromosome 12 Location	Minor Allele	Major Allele	MAF[a]	Without HIV-SN[b] n=46[d]		With HIV-SN[c] n=29[d]		P (Chi²)[e]
P2X7R									
rs10849849	121148592	G	A	0.14	11/46	24%	8/29	28%	0.72
rs1718125	121155216	A	G	0.35	22/44	50%	17/29	59%	0.47
rs208293	121162377	G	A	0.12	11/46	24%	6/29	21%	0.75
rs1169737	121162491	T	C	0.00	0/44	0%	0/24	0%	-
rs1186055[f]	121162726	G	T	0.48	35/46	76%	18/29	62%	0.19
rs208307	121166053	G	C	0.50	34/46	74%	22/29	76%	0.85
rs503720	121167271	G	A	0.39	23/45	51%	21/29	72%	0.07
rs7132846	121177131	T	C	0.04	2/46	4%	3/29	10%	0.37
rs1718119	121177300	C	T	0.44	27/46	59%	20/29	69%	0.37
rs10160951	121180454	G	C	0.18	13/46	28%	11/29	38%	0.38
rs2230912	121184393	G	A	0.04	3/45	7%	3/29	10%	0.67
rs3751142	121184616	A	C	0.13	9/45	20%	7/28	25%	0.62
rs1621388	121184760	C	T	0.43	26/45	58%	19/29	66%	0.51
P2X4R									
rs2686387	121211067	G	C	0.32	24/46	52%	15/29	52%	0.97
rs7298368	121221881	T	C	0.05	3/46	7%	3/29	10%	0.67
rs25644	121228843	G	A	0.06	3/46	7%	4/29	14%	0.42
rs2668252	121230671	C	A	0.36	25/43	58%	13/24	54%	0.75
rs11608486	121232923	C	G	0.04	4/44	9%	2/24	8%	1.00
rs1169719	121233310	A	G	0.11	9/46	20%	8/29	28%	0.42
rs7961979	121233458	A	C	0.13	13/46	28%	4/29	14%	0.17
CAMKK2									
rs1718158	121237098	G	A	0.14	10/46	22%	10/29	34%	0.22
rs10849861	121237504	A	G	0.42	23/46	50%	21/29	72%	0.19
rs1653586	121237772	T	G	0.14	9/46	20%	10/29	34%	0.16
rs1653587	121238429	G	A	0.13	9/46	20%	8/29	28%	0.42
rs11065504[g]	**121242657**	**C**	**G**	**0.10**	**12/45**	**27%**	**2/29**	**7%**	**0.04**
rs2686342	121247385	T	A	0.24	20/46	43%	10/29	34%	0.44
rs3794204	121249212	G	A	0.41	28/46	61%	21/29	72%	0.31
rs7975295	121251298	C	T	0.38	22/46	48%	20/29	69%	0.07
rs2686344	121252745	T	C	0.16	14/46	30%	8/29	28%	0.79
rs1560568	121252784	A	G	0.37	21/45	47%	20/29	69%	0.06
rs1132780	121253293	T	C	0.37	21/45	47%	20/29	69%	0.06
rs7314454	121260982	T	C	0.34	22/45	49%	14/29	48%	0.96
rs11837114	121263634	G	A	0.27	19/41	46%	10/25	40%	0.61
rs1109453	121269518	A	G	0.41	26/45	58%	19/29	66%	0.51
rs3817190	121274274	T	A	0.33	24/46	52%	14/29	48%	0.74
rs9805130	121277592	G	A	0.35	27/46	59%	14/29	48%	0.38
rs7965129	121297604	G	A	0.00	0/45	0%	0/29	0%	-
rs2686367	121297794	A	C	0.27	23/46	50%	13/29	45%	0.66
ANAPC5									
rs113195670	121299178	A	C	0.24	18/43	42%	10/28	36%	0.60
rs2942067	121317758	A	G	0.20	15/45	33%	10/29	34%	0.92
rs2942064	121319857	G	A	0.18	15/46	33%	10/29	34%	0.87
rs2948129	121320159	G	T	0.19	15/46	35%	10/29	34%	0.92
rs2089886	121323973	A	G	0.41	26/45	58%	23/29	79%	0.06
rs74644631	121337408	T	G	0.02	3/43	7%	0/29	0%	0.29
rs11065527	121343210	T	A	0.14	11/45	24%	8/29	28%	0.76
rs2668262	121350299	T	C	0.37	27/45	60%	20/27	74%	0.22
rs1799525	121352124	A	C	0.11	11/45	24%	6/27	22%	0.83
rs7961855	121392140	C	T	0.32	24/46	52%	16/28	57%	0.68

[a] *MAF* – minor allele frequency.
[b] Number of individuals without HIV-SN who carry 1 or 2 copies of the minor allele.
[c] Number of individuals with HIV-SN who carry 1 or 2 copies of the minor allele.
[d] Up to 9 samples failed to reliably genotype for each SNP.
[e] Fisher's Exact test was used where $n < 5$.
[f] Ten SNP which met the criteria for inclusion in multivariate analyses ($p < 0.20$) are shaded.
[g] One SNP in *CAMKK2* which associated with reduced risk of HIV-SN is shown in bold.

60) years and 60% were female (45/75). Nine individuals (12%) were diagnosed with HIV-SN before ART and a further 20 individuals (27%) developed HIV-SN in the following 6-8 months. Pre- and post-ART HIV-SN groups were merged resulting in an overall prevalence of HIV-SN of 38% (29/75). Clinical factors associated with HIV-SN included age, height, body weight, nadir and current CD4 T-cell counts, > 500 copies/ml of HIV RNA and a history of TB ($p < 0.20$; Appendix A) [5]. The optimal logistic regression model included body weight, TB and a low nadir CD4 T-cell count (model $p = 0.0007$; Pseudo $R^2 = 0.18$; Appendix A).

3.2. Five SNPs in P2X7R, CAMKK2 and ANAPC5 associated with HIV-SN

Samples were genotyped for 48 SNP spanning *P2X7R*, *P2X4R*, *CAMKK2* and *ANAPC5*. Two SNP were mono-allelic (rs1169737 and rs7965129). Up to nine individuals could not be genotyped for 21 of the remaining 46 SNP, but all were in HWE and so were assessed for associations with HIV-SN.

Carriage of the minor *CAMKK2* allele, rs11065504*C, associated with lower prevalence of HIV-SN ($p = 0.04$; Table 1). An additional nine SNP across all four genes met the criteria for inclusion in logistic regression analyses ($p < 0.2$; Table 1). This included two SNP in *P2X7R* (rs1186055*G and rs503720*G), one in *P2X4R* (rs7961979*A), five in *CAMKK2* (rs10849861*A, rs1653586*T, rs7975295*C, rs1560568*A and rs1132780*T) and one in *ANAPC5* (rs2089886*A). Three alleles in *CAMKK2* (rs7975295*C, rs1560568*A and rs1132780*T) were in perfect LD and so rs1560568*A and rs1132780*T were excluded. The final model retained body weight, a history of TB, nadir CD4 T-cell counts and five SNP from *P2X7R* (rs503720*G), *CAMKK2 (*rs10849861*A, rs1653586*T, rs11065504*C) and *ANAPC5* (rs2089886*G; model $p < 0.0001$, $n = 69$, Pseudo $R^2 = 0.54$; Table 2). rs11065504*C was again linked with decreased risk of HIV-SN [OR = 0.02, 95% Confidence Interval (95%CI) = 0.00-0.31].

3.3. Three CAMKK2 haplotypes associated with HIV-SN

FastPHASE, applied to each gene individually, yielded 100 haplotypes in *P2X7R*, 17 in *P2X4R*, 153 in *CAMKK2* and 45 in *ANAPC5*. As the estimated frequencies of several haplotypes were low, we restricted our analyses to haplotypes carried by two or more individuals in this population. This resulted in 21 haplotypes in *P2X7R*, 9 in *P2X4R*, 25 in *CAMKK2* and 21 in *ANAPC5*. Haplotype alleles are denoted as "1" (major allele) or "2" (minor alleles) as determined within this population and ordered by chromosomal position as in Table 1.

Nine haplotypes (one in *P2X7R*, one in *P2X4R*, five in *CAMKK2* and two in *ANAPC5*; Table 3) met the criteria for inclusion in logistic regression modelling along with body weight, TB and nadir CD4 T-cell counts. Four of these haplotypes perfectly predicted protection (*P2X7R* haplotype 10 and *CAMKK2* haplotype 8) or risk (*CAMKK2* haplotype 22 and *ANAPC5* haplotype 19) of HIV-SN and were therefore omitted from logistic regression modelling. *P2X7R* haplotype 10 contains no minor alleles and *CAMKK2* haplotype 8 contains three minor alleles – rs11065504*C, rs3714454*T and rs1109453*A. Of which, rs11065504*C independently associated with lower rates of HIV-SN. *CAMKK2* haplotype 22 contained just one minor allele, rs10849861*T, which independently associated with increased risk of HIV-SN. *ANAPC5* haplotype 19 contained five minor alleles, of which, none were linked with HIV-SN in bivariate analyses.

The optimal model retained *CAMKK2* haplotypes 2, 3 and 4 after adjusting for body weight, TB and nadir CD4 T-cell count (model $p = 0.0005$, $n = 71$, Pseudo $R^2 = 0.26$; Table 2). All three *CAMKK2* haplotypes in the optimal model associated with increased risk of HIV-SN (OR = 4.44-7.79; Table 2). No minor alleles were common across all three haplotypes although they share three major alleles: rs11065504*G, rs7965129*A and rs2686367*C. Of which, the minor "C" allele of rs11065504 associated with lower rates of HIV-SN in bivariate and multivariate analyses (Table 1 and 2).

Table 2
CAMKK2 SNP and haplotypes associated with HIV-SN in Africans.

Variable	Odds Ratio	P	95% Confidence Interval
SNP Model[a]: n = 69,[b]p < 0.0001, Pseudo R^2 = 0.54			
Body Weight (kg)	1.07	0.031	1.01–1.13
History of Tuberculosis	11.28	0.071	0.81–156
Nadir CD4 T-cells/μl	1.00	0.007	0.98–1.00
rs503720*G (*P2X7R*)	133	0.002	6.47–2757
rs10849861*A (*CAMKK2*)	5.99	0.050	1.00–35.9
rs1653586*T (*CAMKK2*)	67.8	0.004	3.80–1210
rs11065504*C (*CAMKK2*)	0.02	0.006	0.00–0.31
rs2089886*A (*ANAPC5*)	6.68	0.088	0.76–58.9
Haplotype Model[c]: n = 71,[d]p = 0.0005, Pseudo R^2 = 0.26			
Body Weight (kg)	1.03	0.032	1.00–1.07
History of Tuberculosis	5.81	0.076	1.16–29.1
Nadir CD4 T-cells/μl	0.99	0.049	0.99–1.00
CAMKK2 Haplotype 2	2.82	0.153	0.68–11.7
CAMKK2 Haplotype 3	3.42	0.106	0.77–15.2
CAMKK2 Haplotype 4	6.85	0.110	0.65–72.7
Haplotypes which aligned perfectly with the presence or absence of HIV-SN[e]			
P2X7R Haplotype 10	Never found in individuals with HIV-SN		
CAMKK2 Haplotype 8	Never found in individuals with HIV-SN		
CAMKK2 Haplotype 22	Only found in individuals with HIV-SN		
ANAPC5 Haplotype 19	Only found in individuals with HIV-SN		

[a] The optimal logistic regression model considering demographic variables and SNP.
[b] Excluding samples with missing demographic, clinical and/or genotype data.
[c] The optimal logistic regression model considering demographic variables and haplotypes.
[d] Excluding samples with missing demographic or clinical data, or individuals carrying haplotypes aligned perfectly with the presence or absence of HIV-SN.
[e] Haplotypes excluded from multivariate analyses as they aligned perfectly with risk or protection of HIV-SN.

Haplotype networks were built for each gene using all haplotypes derived by fastPHASE to determine if haplotypes associating with HIV-SN were clustered and to permit analyses of rarer haplotypes but no distinct haplogroups were identified for any gene (Appendix A).

4. Discussion

This study investigated HIV-SN in Southern African patients before ART and after 6-8 months on ART which excluded the neurotoxic drug stavudine. We assessed associations between HIV-SN and polymorphisms in *P2X7R*, *P2X4R*, *CAMKK2,* and *ANAPC5*, as alleles of these genes affected HIV-SN in patients receiving stavudine [29]. Our inclusion and exclusion criteria minimised alternative causes of neuropathy, but a limitation of our study is the modest number of participants recruited. This limited our ability to correct for multiple comparisons and our power to investigate associations between HIV-SN and infrequent SNP and haplotypes. The large confidence intervals obtained in some regression models reflect the resulting uncertainty. Nonetheless we confirm that HIV-SN remains prevalent and can develop before or on stavudine-free ART, and establish a role for *CAMKK2* SNP and haplotypes in HIV-SN in patients treated without stavudine.

HIV-SN was diagnosed in nine individuals (12%) before commencing ART and an additional 20 cases (27%) re-assessed at 6-8 months. The timing suggests a role for HIV itself and/or immune recovery on ART in HIV-SN. Some individuals experience acute inflammation shortly after initiating ART (known as immune restoration inflammatory syndrome, IRIS). This has been linked to HIV-SN and neuropathic pain [18,39]. However, signs of HIV-SN may also persist or evolve when HIV RNA is undetectable and CD4 T-cells are replenished [40]. A direct role for HIV remains plausible as animal and human *ex*

Table 3
Nine haplotypes associated weakly with HIV-SN.

Haplotype Number	Haplotype Alleles	Without HIV-SN [a] n=46		With HIV-SN [b] n=29		P (Chi^2) [c]
P2X7R						
1	1111221111111	10	22%	4	14%	0.55
2	1211121111111	7	15%	5	17%	0.82
3	1111121111111	7	15%	7	24%	0.33
4	1211121112111	6	13%	5	17%	0.62
5	1111112121112	5	11%	2	7%	0.70
6	2211212121112	4	9%	3	10%	1.00
7	1111212121122	6	13%	1	3%	0.24
8	1121212121112	6	13%	2	7%	0.47
9	1111211121112	4	9%	3	10%	1.00
10[d,e]	1111111111111	5	11%	0	0%	0.15
11	1111211111111	2	4%	1	3%	1.00
12	1211112121112	1	2%	3	10%	0.29
13	1111212121112	1	2%	1	3%	1.00
14	1111121112111	2	4%	1	3%	1.00
15	2211112112111	1	2%	2	7%	0.56
16	1111111112111	2	4%	2	7%	0.64
17	1111221111211	2	4%	1	3%	1.00
18	2211211121112	1	2%	1	3%	1.00
19	2211112121122	1	2%	2	7%	0.56
20	1121222221122	2	4%	0	0%	0.52
21	2211121112111	2	4%	0	0%	0.52
P2X4R						
1	1111111	33	72%	24	83%	0.28
2	2112111	11	24%	4	14%	0.38
3	2112121	9	20%	8	28%	0.42
4	1112112	8	17%	1	3%	0.14
5	2222111	3	7%	3	10%	0.67
6	2111112	5	11%	1	3%	0.40
7	1112212	2	4%	1	3%	1.00
8	1112211	2	4%	0	0%	0.52
9	1111212	1	2%	1	3%	1.00
CAMKK2						
1	121111221222121111	10	22%	7	24%	0.81
2	212211111111112211	7	15%	8	28%	0.19
3	111112112112121111	5	11%	7	24%	0.13
4	121111221221212211	2	4%	4	14%	0.19
5	111111211111211111	2	4%	1	3%	1.00
6	121111121221121112	3	7%	3	10%	0.67
7	121111121222121111	1	2%	1	3%	1.00
8[e,g]	1111[2]1111112121111	6	13%	0	0%	0.08
9	111111111111211111	3	7%	0	0%	0.28
10	1111[2]1111111111111	4	9%	1	3%	0.64
11	111111111111212212	2	4%	2	7%	0.64

(*continued on next page*)

Table 3 (*continued*)

12	121111221222121112	1	2%	1	3%	1.00
13	121111221221112211	2	4%	1	3%	1.00
14	121111221222111212	1	2%	2	7%	0.56
15	121111221221112212	4	9%	2	7%	1.00
16	111112112111121111	1	2%	1	3%	1.00
17	111111211111211112	2	4%	1	3%	1.00
18	121112112112121111	2	4%	0	0%	0.52
19	121111221221211111	1	2%	2	7%	0.56
20	11112[2]1111111112211	2	4%	0	0%	0.52
21	121111211111111112	1	2%	1	3%	0.62
22[f]	121111111111111111	0	0%	2	7%	0.15
23	111112211111211111	2	4%	1	3%	1.00
24	111111111111211212	2	4%	0	0%	0.52
25	111112211111211212	2	4%	0	0%	0.52
ANAPC5						
1	1111111111	20	44%	10	34%	0.44
2	1111211211	9	20%	8	28%	0.42
3	2111111111	10	22%	6	21%	0.91
4	2111211211	4	9%	3	10%	1.00
5	1111211111	4	9%	4	14%	0.70
6	1111111112	3	7%	2	7%	1.00
7	2111211212	6	13%	4	14%	1.00
8	1111211212	2	4%	5	17%	0.10
9	1222112111	3	7%	2	7%	1.00
10	1222112121	3	7%	1	3%	1.00
11	1222112212	3	7%	2	7%	1.00
12	1111211121	3	7%	0	0%	0.28
13	1222111121	2	4%	1	3%	1.00
14	1111211112	2	4%	0	0%	0.52
15	1222112122	1	2%	2	7%	0.56
16	1222112211	2	4%	0	0%	0.52
17	1222111122	2	4%	1	3%	1.00
18	1111111212	2	4%	0	0%	0.52
19[f]	1222112112	0	0%	2	7%	0.15
20	1112111112	1	2%	1	3%	1.00
21	1211111112	2	4%	0	0%	0.52

[a] The number of individuals without HIV-SN who carry 1 or 2 copies of the haplotype.
[b] The number of individuals with HIV-SN who carry 1 or 2 copies of the haplotype.
[c] Fisher's Exact test was used when $n < 5$.
[d] Nine haplotypes meeting criteria for inclusion in logistic regression models ($p < 0.20$) are shaded.
[e] Haplotypes never found in individuals with HIV-SN.
[f] Haplotypes only found in individuals with HIV-SN.

vivo studies demonstrate a role for inflammation initiated by the HIV envelope glycoprotein 120 [41,42]. Indeed, significant clinical risk factors include low nadir CD4 T-cell counts, which are a permanent marker of a history of severe HIV disease (Appendix A). The data are consistent with studies prior to the advent of combination ART [43–45] and with our findings in Indonesians treated for 4.4 (1.1-12.7) years without stavudine [2]. Participants here were treated without stavudine, but it remains possible that other NRTIs may promote HIV-SN. Most participants (72/75) received tenofovir, which has been implicated in the development of peripheral neuropathy in mice [46]. However, studies in humans report no associations between tenofovir use and HIV-SN [2,47,48]. Further studies addressing the impact of HIV, immune recovery and next generation NRTIs on peripheral nerves are warranted.

Genetic analyses generated two models predicting risk of HIV-SN. In the optimal model considering haplotypes, three common *CAMKK2* haplotypes (*CAMKK2* haplotype 2, 3 and 4) associated with HIV-SN after adjusting for lower nadir CD4 T-cell count, prior tuberculosis and greater body weight (model $p = 0.0007$; Pseudo $R^2 = 0.26$; Table 2). No minor alleles were common across all three haplotypes but two minor alleles (rs3817190*T and r9805130*G) were shared by *CAMKK2* haplotypes 2 and 4, and so warrant consideration. rs3817190 is a non-synonymous SNP, changing a threonine (rs3817190*T) to serine (rs3817190*A) in exon 1, reducing the ability of CaMKK2 to function

autonomously [20]. However in Indonesians, rs3817190*A is the minor allele and is found in a haplotype associated with increased risk of HIV-SN [30]. In South Africans, rs3817190*T is the minor allele in a haplotype associated with risk. Moreover, neither rs3817190*T nor r9805130*G associated with HIV-SN in bivariate analyses in South Africans ($p = 0.74$ and 0.38; Table 2) so these are probably not causative alleles. Three major alleles were common between all three risk haplotypes – rs11065504*G, rs7965129*A and rs2686367*C. rs7965129 is monoallelic and rs2686367 did not associate with HIV-SN in bivariate analyses here ($p = 0.66$; Table 2) or in Indonesians ($p = 0.51$; [30]). The minor "C" allele of rs11065504 associated with lower risk in bivariate analyses (OR = 0.02, 95% CI = 0.00-0.31; Table 1) and was retained in the logistic regression. This SNP warrants further consideration.

In the optimal model considering SNP alleles, rs503720*G in *P2X7R*, rs10849861*A, rs1653586*T and rs11065504*C in *CAMKK2*, and rs2089886*A in *ANAPC5* associated with HIV-SN after adjusting for body weight, nadir CD4 T-cell counts and TB (model $p < 0.0001$, $n = 69$, pseudo $R^2 = 0.54$; Table 2). These SNP demonstrate inconsistent associations and are therefore unlikely to play a direct role in HIV-SN. For example, rs503720*G and rs2089886*A associated with HIV-SN in Africans, but showed no associations in Indonesians [30]. Furthermore, no *P2X7R* and *ANAPC5* haplotypes were retained in the optimal haplotype model, and just one *P2X7R* and *ANAPC5* haplotype aligned perfectly with risk or protection of HIV-SN but did not contain rs503720*G or rs2089886*A.

rs11065504*C which associated with decreased risk (OR = 0.02, 95%CI = 0.00–0.31) and rs10849861*A with increased risk of HIV-SN (OR = 5.99, 95CI% = 1.00–35.9; Table 2) demonstrated opposing associations in Indonesians, both individually or within haplotypes, and rs1653586*T does not associate with HIV-SN in Indonesians [30]. This suggests that these *CAMKK2* alleles also do not play a direct role in HIV-SN. However, these three alleles are consistently found within haplotypes associated with altered risk of HIV-SN here in Africans. rs11065504*C is one of three minor alleles found in *CAMKK2* haplotype 8, which aligned with protection from HIV-SN (Table 3). Furthermore, the corresponding major "risk" allele (rs11065504*G) was contained within all three haplotypes associated with increased risk of HIV-SN (*CAMKK2* haplotypes 2, 3 and 4; Table 2). rs10849861*A is the only minor allele in *CAMKK2* haplotype 22, which aligned perfectly with risk of HIV-SN (Table 3), and is included in *CAMKK2* haplotype 4 (Table 3). Lastly, rs1653586*T was contained in *CAMKK2* haplotype 2 which associated with risk of HIV-SN (Table 2). It is plausible that while these alleles may not directly drive HIV-SN, they may be in LD and co-inherited with the causative allele/s.

Our results show clearer associations between HIV-SN and polymorphisms and haplotypes of *CAMKK2* than with *P2X7R*, *P2X4R,* and *ANAPC5*. While all four genes are involved in inflammation, *CAMKK2* is crucial in neuronal growth and repair pathways. Associations with *CAMKK2* may reflect an individual's ability to recover from neuronal insults, so pharmacological activation or inhibition of CaMKK2 and/or its substrates may prevent or revert pathological features of HIV-SN. This is supported in a rodent model of diabetic peripheral neuropathy, whereby activation of AMPK (a CaMKK2 substrate) with anti-muscarinic drugs overcame mitochondrial dysfunction [26]. Treatment prevented or reversed markers of peripheral neuropathy including loss of sensory nerve terminals, thermal hyperalgesia and decreased nerve conduction.

Overall, this study confirms that HIV-SN remains a clinically important issue affecting HIV+ individuals prior to and shortly after commencing ART without stavudine. Furthermore, our results confirm a role for polymorphisms in *CAMKK2* in the pathogenesis of HIV-SN independent of stavudine, but critical genotypes differ between ethnicities [30] so the causative SNP have not been identified. Our approach utilising multivariable analyses identifies the strongest determinants of risk. Replications of this study in large independent cohorts, defined by treatment status and ethnicity, are warranted to confirm our findings and to elucidate underlying mechanisms.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jns.2020.116987.

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ONLINE SUPPLEMENT 1

Supplementary Table 1. Demographic and clinical variables associate with HIV-SN in Africans

Variable	With HIV-SN (n=29)	Without HIV-SN (n=46)	P value
Age (years)	40 (24-60)	37 (19-58)	0.11
Height (cm)	**168 (147-179)**	**163 (135-186 (n=45)**	**0.03**
Weight (kg)	**66 (45-112)**	**55 (35-110) n=44**	**0.03**
Current CD4 T-cells/µl	221 (22-685)	300 (8-832)	0.06
Nadir CD4 T-cells/µl	**107 (4-575)**	**223 (8-771)**	**0.002**
HIV RNA >500 copies/ml	21/29 (72%)	25/46 (54%)	0.12
History of Tuberculosis	6/28 (29%)	3/45 (7%)	0.08[c]
Female Gender	15/29 (52%)	30/46 (65%)	0.25

Variables significantly associated with HIV-SN are in bold ($p<0.05$). All demographic variables achieving $p<0.20$ were included in logistic regression modelling.

[a] Mann-Whitney test used to assess all continuous variables – Median (range)

[b] χ^2 test used to assess dichotomous variables – Proportion (%)

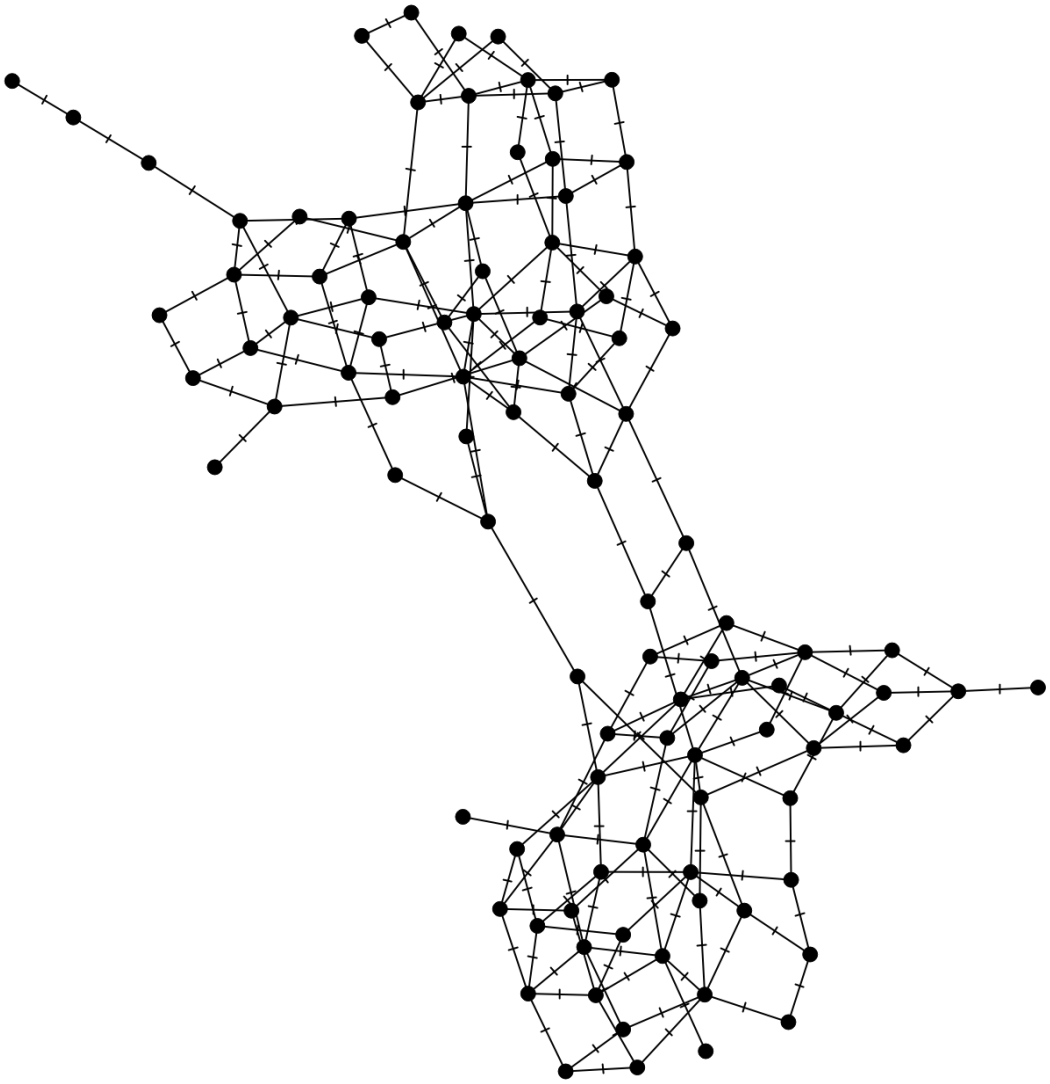
[c] Fisher's Exact test used where n<5

Supplementary Table 2. Logistic regression modelling identifies demographic and clinical variables independently associating with HIV-SN in Africans

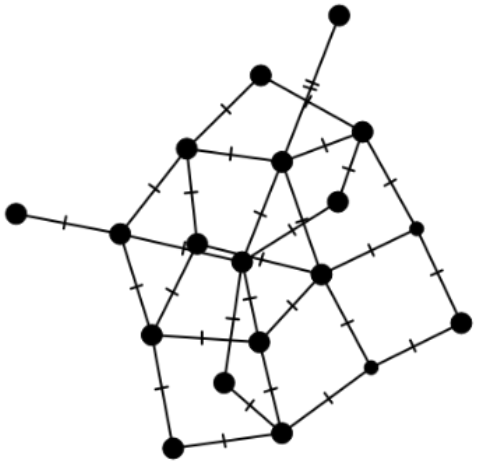
Variable	Odds Ratio	P Value	95% CI
Optimal Model: n=71[a], p=0.0007, Pseudo R^2=0.18			
Weight (kg)	1.04	0.03	1.00-1.08
History of Tuberculosis	4.26	0.07	0.90-20.03
Nadir CD4 T-cells/µl	1.00	0.03	0.99-1.00

[a] excluding samples with missing demographic, clinical and/or genotype data

ONLINE SUPPLEMENT 2

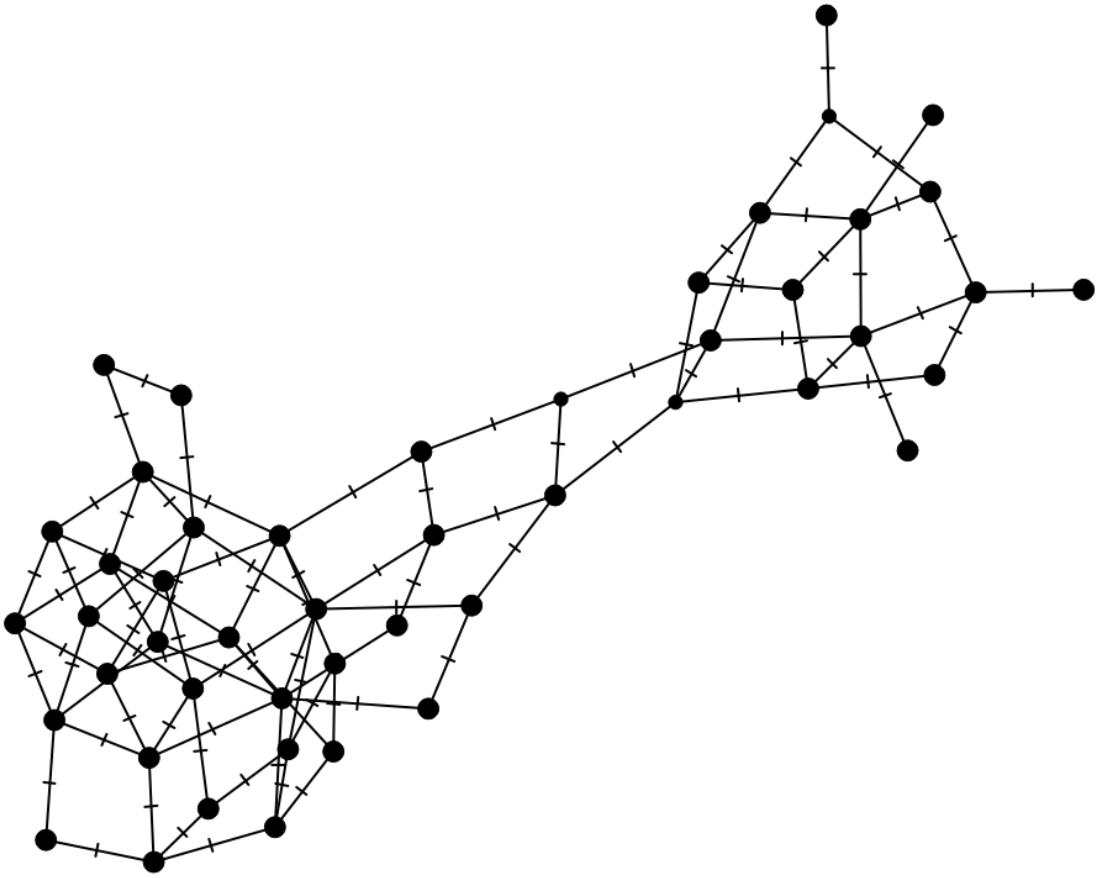


Supplementary Figure 1. No clear *P2X7R* haplogroups were identified
The *P2X7R* haplotype network was constructed in PopART using the Median-Joining method and an epsilon value of 0. The network includes 100 *P2X7R* haplotypes which were derived from the 13-SNP genotypes of the 75 Southern African using fastPHASE. No distinct haplotype clusters can be seen.



Supplementary Figure 2. No clear *P2X4R* haplogroups were identified
The *P2X4R* haplotype network was constructed in PopART using the Median-Joining method and an epsilon value of 0. The network includes 17 *P2X4R* haplotypes which were derived from the 7-SNP genotypes of the 75 Southern African using fastPHASE. No distinct haplotype clusters can be seen.

Supplementary Figure 3. No clear *CAMKK2* haplogroups were identified
The *CAMKK2* haplotype network was constructed in PopART using the Median-Joining method and an epsilon value of 0. The network includes 160 *CAMKK2* haplotypes which were derived from the 18-SNP genotypes of the 75 Southern African using fastPHASE. No distinct haplotype clusters can be seen.

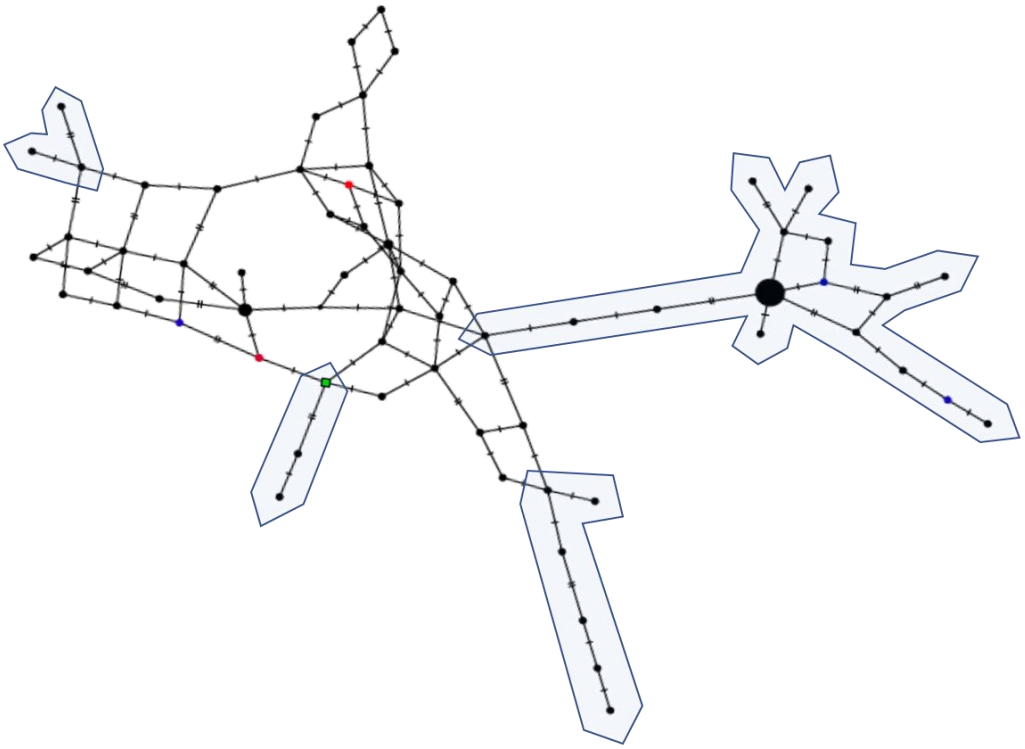


Supplementary Figure 4. No clear *ANAPC5* haplogroups were identified
The *ANAPC5* haplotype network was constructed in PopART using the Median-Joining method and an epsilon value of 0. The network includes 45 *ANAPC5* haplotypes which were derived from the 11-SNP genotypes of the 75 Southern African using fastPHASE. No distinct haplotype clusters can be seen

Chapter 4

Polymorphisms in *CAMKK2* associate with susceptibility to sensory neuropathy in HIV patients treated without stavudine

Polymorphisms in the P2X-block associated with HIV-SN in South Africans treated with and without stavudine, confirming a role independent of mechanisms invoked by stavudine. However, genotypes identified differed between the two South African cohorts so the causative alleles may differ in the manifestation of HIV-SN without stavudine. To help identify critical genotypes, I next investigated associations between the P2X-block and HIV-SN in Indonesians treated without stavudine.



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Polymorphisms in *CAMKK2* associate with susceptibility to sensory neuropathy in HIV patients treated without stavudine

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Abstract

HIV-associated sensory neuropathy (HIV-SN) is a debilitating neurological complication of HIV infection potentiated by the antiretroviral drug stavudine. While stavudine is no longer used, HIV-SN now affects around 15% of HIV+ Indonesians. Here, we investigate whether polymorphisms within the *P2X*-block (*P2X4R*, *P2X7R*, *CAMKK2*) and/or *ANAPC5* mark susceptibility to HIV-SN in this setting. As polymorphisms in these genes associated with HIV-SN in African HIV patients receiving stavudine, the comparison can identify mechanisms independent of stavudine. HIV patients who had never used stavudine ($n = 202$) attending clinics in Jakarta were screened for neuropathy using the AIDS Clinical Trials Group Brief Peripheral Neuropathy Screen. Open-array technology was used to type 48 polymorphisms spanning the four genes. Haplotypes were derived for each gene using fastPHASE. Haplogroups were constructed with median-joining methods. Multivariable models optimally predicting HIV-SN were based on factors achieving $p < 0.2$ in bivariate analyses. Minor alleles of three co-inherited polymorphisms in *CAMKK2* (rs7975295*C, rs1560568*A, rs1132780*T) associated with a reduced prevalence of HIV-SN individually and after adjusting for lower CD4 T cell count and viremia ($p = 0.0002$, pseudo $R^2 = 0.11$). The optimal model for haplotypes linked HIV-SN with viremia and lower current CD4 T cell count, plus *CAMKK2* haplotypes 6 and 11 and *P2X7R* haplotypes 2 and 12 ($p = 0.0002$; pseudo $R^2 = 0.11$). *CAMKK2* haplogroup A (includes 16 haplotypes and all instances of rs7975295*C, rs1560568*A, rs1132780*T) associated with reduced rates of HIV-SN ($p = 0.02$, OR = 0.43 CI = 0.21–0.88). These findings support a protective role for these three alleles, suggesting a role in the pathogenesis of HIV-SN that is independent of stavudine.

Keywords HIV · Sensory neuropathy · *CAMKK2* · Single nucleotide polymorphisms · Indonesia

Introduction

HIV-associated sensory neuropathy is a debilitating neurological complication of HIV infection and antiretroviral therapy (ART) (Evans et al. 2011; Keswani et al. 2002; Smyth et al. 2007; Wulff et al. 2000). Neurotoxic ART such as stavudine has been replaced with safer therapies, reducing the prevalence of HIV-SN from 34% to 15% in HIV+ Indonesians (Affandi et al. 2008; Octaviana et al. 2018). HIV-SN can impact an individual's ability to work and their quality of life. No interventions prevent HIV-SN progression (Ellis et al. 2010; Phillips et al. 2010), so a better understanding of the underlying mechanisms will have clinical value.

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HIV-SN is predominantly a small-fibre neuropathy, affecting peripheral C-fibres with loss of neurons in the dorsal root ganglia (DRG), degeneration of long axons and loss of primary afferent terminals in the epidermis of the feet and hands (Shikuma et al. 2015). Signs of an inflammatory pathology include infiltration of macrophages and expression of chemokine receptors in the affected epidermis (Mountford et al. 2018; Polydefkis et al. 2002) and associations with genotypes of *TNFA* and neighbouring genes (Hendry et al. 2016; Wadley et al. 2015). The mechanisms driving the neuronal death and inflammation are not fully understood, but P2X7R, P2X4R and CaMKK2 are plausible candidates.

P2X7R and P2X4R are purinergic receptors involved in inflammatory pathways and excitatory neurotransmission following activation by ATP (Tsuda et al. 2012). P2X7R knockout mice demonstrate reduced mechanical and thermal perception whereas tactile allodynia is reduced following administration of P2X7R inhibitors in rats in a model of neuropathic pain (Chessell et al. 2005). Additionally, P2X7R induces pro-inflammatory cytokines including TNFα (Kawasaki et al. 2008). Stimulation of P2X4R in hyperactive microglia triggers tactile allodynia (Tsuda et al. 2003). Activation of P2X4R stimulates prostaglandin E2 which in turn sensitises peripheral nociceptors increasing the responsiveness of sensory neurons in the periphery (Lin et al. 2006).

CaMKK2 (calcium/calmodulin kinase kinase 2) phosphorylates AMPK (AMP-activated protein kinase), SIRT1 (sirtuin 1), CaMKIV and CaMKI (calcium/calmodulin kinase 4 and 1) (Wen et al. 2013); Kokubo et al. 2009; Racioppi and Means 2012). AMPK activation is associated with reduced inflammatory and macrophage responses (Racioppi et al. 2012; Zhang et al. 2011). SIRT1 regulates axonal regeneration, protects neurons from oxidative stress and promotes dendrite arborisation (Codocedo et al. 2012; Li et al. 2008; Liu et al. 2013). CaMKIV activation upregulates NFkB and cAMP response element-binding protein (CREB) which stimulates brain-derived neurotrophic factor (BDNF) promoting neuronal growth and survival (Cao and DeLeo 2008; Racioppi and Means 2012; Wayman et al. 2008). BDNF has been implicated in neuropathic pain (Coull et al. 2005; Ulmann et al. 2008). Activated CaMKI regulates axonal growth cone morphology and outgrowth, dendrite arborisation and synapse formation (Ageta-Ishihara et al. 2009; Wayman et al. 2008).

P2X7R, *P2X4R* and *CAMKK2* (the "*P2X*-block") are neighbouring genes located in a region of high linkage disequilibrium (LD) in chromosome 12—spanning approximately 165 kb. Single nucleotide polymorphisms (SNPs) and haplotypes from the *P2X*-block associated with HIV-SN in Southern African HIV+ patients receiving stavudine-based ART (Goullee et al. 2016). An optimal multivariable model linked rs208307*G in *P2X7R*, rs2668252*C and rs1169719*A in *P2X4R* and rs1560568*A, rs7975295*C and rs2686387*A in *CAMKK2*, plus age and height, with increased risk of HIV-SN. A second model linked HIV-SN status with age, height, a *P2X7R* haplotype and three *CAMKK2* haplotypes, which associated with reduced risk of HIV-SN despite carrying rs1560568*A and rs7975295*C. As these SNPs had opposing associations when assessed independently and in haplotypes, the causal SNP/s may lay outside the panel tested.

Topologically associated domains (TADs) have been identified on chromosome 12 in astrocytes from the cerebellum and spinal cord. The larger TAD spans 120600001–121840000 bp and encompasses 30 genes, including the *P2X*-block and ending at the 5′ border of *ANAPC5* (https://www.encodeproject.org/experiments/; Online Resource 1). *ANAPC5* lies upstream of *CAMKK2* and encodes the anaphase promoting complex 5 (AnapC5), one of over 12 subunits of the anaphase-promoting complex (APC) — an E3 ubiquitin ligase which tags A and B cyclins, marking them for destruction by the 26S proteasome. This permits progression of the cell cycle from metaphase into anaphase and so promotes mitosis (Peters 2006; Thornton and Toczyski 2006; Zhou et al. 2016). Replication of neurons would disrupt signal transduction so re-entry into the cell cycle results in apoptosis (Kaul et al. 2001; Windebank and McDonald 2002). Altered function or expression of *ANAPC5* may alter APC activity and result in neuronal death characteristic of HIV-SN. AnapC5 also interacts with IL-17RA (Ho et al. 2013), the receptor for the IL-17 inflammatory cytokine, reducing downstream signalling which may impair defence against infections and modify inflammatory diseases including psoriasis, rheumatoid arthritis and multiple sclerosis (Chang et al. 2011; Genovese et al. 2010; Papp et al. 2012). Hence, SNPs in *ANAPC5* may also contribute to HIV-SN via inflammatory pathways.

Here, we investigate whether SNPs within the *P2X*-block and *ANAPC5* mark susceptibility to HIV-SN in Indonesian HIV+ patients who have never received neurotoxic stavudine (Octaviana et al. 2018).

Materials and methods

Participants

HIV-positive adults who had used ART for at least 12 months but who had never been exposed to stavudine were screened for neuropathy at POKDISUS HIV Care Clinic, Cipto Mangunkusumo Hospital, Jakarta, Indonesia. Patients with any history of another condition that might be associated with a neuropathy or any condition preventing the patient from being able to provide informed consent were excluded. Neuropathy was assessed using the AIDS Clinical Trials Group Brief Peripheral Neuropathy Screen (ACTG-BPNS) and defined as present if the individual had one or more of

the lower limb neuropathic symptoms (pain, aching or burning, pins and needles or numbness), plus absent ankle reflexes or reduced vibration sense at the great toe (vibration of a 128-Hz tuning fork felt for 10 s or less). We did not diagnose neuropathy in patients with only asymptomatic neuropathic signs, as the presence of both symptoms and signs on the ACTG-BPNS tool better associates with impaired peripheral nerve function and pathology (Cherry et al. 2005). Demographic associations with HIV-SN in the parent cohort have been described previously (Octaviana et al. 2018). DNA samples were available from 185 patients and an additional 9 HIV-SN and 8 age and gender-matched Indonesian HIV+ patients without HIV-SN recruited in 2012 at the same clinic and who met the inclusion criteria. The study was approved by the Ethics Committee of the Faculty of Medicine, Universitas Indonesia. Written and informed consent was obtained.

Genotyping and Haplotyping

DNA was extracted from EDTA-blood samples using Favorprep Blood Genomic DNA Extraction Mini Kit (Favorgen, Taiwan). Samples were adjusted to 50 ng/μL, diluted 1:1 with TaqMan® OpenArray™ Genotyping Master Mix and genotyped for 48 SNPs across *P2X7R*, *P2X4R*, *CAMKK2* and *ANAPC5* using custom TaqMan® OpenArray™ Real-Time PCR Plates using the QuantStudio 12 K Flex Real-Time PCR System (Life Technologies, NY). Genotypes were assigned manually using TaqMan® Genotyper Software and assessed for conformance with Hardy-Weinberg Equilibrium (HWE). All SNPs met HWE criteria except for five SNPs that were monoallelic in Indonesians (rs1169737, rs10160951, rs1169719, rs11837114 and rs7965129). These were retained in our analyses. Haplotypes and estimated population frequencies were derived for each gene individually using fastPHASE (Scheet and Stephens 2006) with the default parameters and sampled from the observed genotypes an additional 5000 times per sample. Haplotypes with an estimated frequency less than 1% were excluded from analyses. Haplotype networks were constructed for *P2X7R*, *P2X4R* and *CAMKK2* with median-joining methods (Bandelt et al. 1999) using PopART v1.7 (Population Analysis with Reticulate Trees, Otago, New Zealand; http://popart.otago.ac.nz). Non-synonymous SNPs linked with HIV-SN were assessed with the SIFT (sorting intolerant from tolerant) web server to predict their impact on protein function (https://sift.bii.a-star.edu.sg/www/SIFT_dbSNP.html; Sim et al. 2012).

Statistical analyses

Stata 12 (StataCorp, TX) was used to assess bivariate associations between HIV-SN and demographic and clinical variables, SNPs and haplotypes using *t* tests, Mann-Whitney tests, Chi^2 or Fisher's exact tests, as appropriate. No corrections were made for multiple comparisons in this exploratory study. As many SNPs and haplotypes occurred at low frequencies, all variables which showed weak ($p = 0.05$–0.2) or significant ($p < 0.05$) associations were included in logistic regression modelling. Optimal logistic regression models were determined with a stepwise removal process.

Results

Markers of severe and persistent HIV disease associated with HIV-SN

In the parent cohort ($n = 197$) described previously, HIV-SN was associated with poor control of HIV replication on ART (Octaviana et al. 2018). With the additional 17 patients, 17% (35/202) had HIV-SN and 28.7% (58/202) were female, with a median (range) age of 35 years (19–60), 434 (44–1166) CD4 T cells/μl and 0 (0–121,000) copies HIV RNA/ml at the time of assessment. Demographic factors associated with HIV-SN in bivariate analyses and included in the logistic regression modelling included current CD4 T cell count ($p = 0.006$), current viremia (> 500 copies of HIV RNA/ml; $p = 0.004$), a nadir CD4 T cell count < 200/μl ($p = 0.02$) and a history of tuberculosis ($p = 0.14$). The current viral load and/or CD4 T cell count was not available for seven patients, so the logistic regression models were based on 195 patients. The final model identified current viremia and a lower CD4 T cell count as the independent clinical associations with HIV-SN ($p = 0.0006$; pseudo $R^2 = 0.08$).

Three SNPs in *CAMKK2* associated with reduced prevalence of HIV-SN

Minor alleles of three SNPs in *CAMKK2* (rs7975295*C, rs1560568*A and rs1132780*T) associated with a reduced risk of HIV-SN in univariate analyses ($p < 0.05$; OR = 0.44; 95% CI = 0.21–0.94; Table 1). However, the minor alleles were invariably co-inherited so only rs1560568*A was included in logistic regression modelling. We also included rs25644*G in *P2X4R* and rs10849861*A in *CAMKK2* (as they achieved $p < 0.2$), plus current CD4 T cell count and viremia. The optimal model identified carriage of rs1560568*A (marking rs7975295*C and rs1132780*A) as an independent association with HIV-SN, after adjustment for a lower CD4 T cell count and viremia ($p = 0.0002$, pseudo $R^2 = 0.11$; Table 3). In this model, carriage of rs1560568*A was again associated with a reduced prevalence of HIV-SN (OR = 0.43, 95% CI = 0.19–0.95) so at least one of the three alleles may be protective.

Table 1 Three SNPs in *CAMKK2* significantly associate with reduced risk of HIV-SN

RSID	Chromosome 12 Location	Minor Allele	Major Allele	MAF[a]	Without HIV-SN[b,d]		With HIV-SN[c,d]		P (Chi^2)[e]
P2X7R									
rs10849849	121148592	G	A	0.32	88/167	53%	17/35	49%	0.66
rs1718125	121155216	A	G	0.43	117/167	70%	21/35	60%	0.24
rs208293	121162377	G	A	0.50	123/167	74%	26/35	74%	0.94
rs1169737	121162491	T	C	0.00	0/167	0%	0/35	0%	1.00
rs1186055	121162726	T	G	0.38	101/165	61%	19/35	54%	0.45
rs208307	121166053	G	C	0.17	54/165	33%	8/35	23%	0.25
rs503720	121167271	A	G	0.18	56/167	34%	9/34	26%	0.42
rs7132846	121177131	T	C	0.22	65/166	39%	13/35	37%	0.82
rs1718119	121177300	T	C	0.21	59/165	36%	11/35	31%	0.63
rs10160951	121180454	G	C	0.00	0/166	0%	0/35	0%	1.00
rs2230912	121184393	G	A	0.01	4/167	2%	0/34	0%	1.00
rs3751142	121184616	A	C	0.22	66/167	40%	13/35	37%	0.79
rs1621388	121184760	T	C	0.21	58/166	35%	11/35	31%	0.69
P2X4R									
rs2686387	121211067	G	C	0.41	109/167	65%	20/35	57%	0.36
rs7298368	121221881	T	C	0.41	107/166	64%	19/35	54%	0.26
rs25644	121228843	G	A	0.31	88/164	54%	14/35	40%	0.14
rs2668252	121230671	C	A	0.43	111/166	67%	22/35	63%	0.65
rs11608486	121232923	C	G	0.01	2/162	1%	0/34	0%	1.00
rs1169719	121233310	A	G	0.00	0/166	0%	0/35	0%	1.00
rs7961979	121233458	A	C	0.02	6/167	4%	0/35	0%	0.59
CAMKK2									
rs1718158	121237098	G	A	0.10	27/166	16%	8/35	23%	0.35
rs10849861	121237504	A	G	0.32	92/166	55%	14/35	40%	0.10
rs1653586	121237772	T	G	0.10	27/166	16%	8/35	23%	0.35
rs1653587	121238429	G	A	0.08	23/167	14%	7/35	20%	0.35
rs11065504	121242657	C	G	0.30	84/165	51%	16/35	46%	0.58
rs2686342	121247385	T	A	0.25	66/166	40%	17/35	49%	0.34
rs3794204	121249212	G	A	0.47	120/166	72%	22/35	63%	0.27
rs7975295	121251298	C	T	0.34	100/166	60%	14/35	40%	0.028
rs2686344	121252745	T	C	0.24	64/166	39%	17/35	49%	0.27
rs1560568	121252784	A	G	0.34	101/167	60%	14/35	40%	0.026
rs1132780	121253293	T	C	0.34	100/165	61%	14/35	40%	0.037
rs7314454	121260982	T	C	0.04	15/165	9%	2/35	6%	0.74
rs11837114	121263634	G	A	0.00	0/162	0%	0/33	0%	1.00
rs1109453	121269518	A	G	0.07	21/165	13%	3/35	9%	0.77
rs3817190	121274274	A	T	0.18	57/167	34%	12/35	34%	0.99
rs9805130	121277592	A	G	0.19	59/167	35%	12/35	34%	0.94
rs7965129	121297604	G	A	0.00	0/166	0%	0/35	0%	1.00
rs2686367	121297794	C	A	0.13	38/163	23%	10/35	29%	0.51

[a] *MAF* – minor allele frequency
[b] Number of individuals without HIV-SN who carry 1 or 2 copies of the minor allele
[c] Number of individuals with HIV-SN who carry 1 or 2 copies of the minor allele
[d] Up to 6 samples failed to genotype for each SNP
[e] Fisher's Exact test was used where n<5
[f] Three SNPs in CAMKK2 which significantly associate with reduced risk of HIV-SN are in bold
[g] Five SNPs which met the criteria for inclusion in logistic regression modelling (p<0.2) are shaded

Two *CAMKK2* and two *P2X7R* haplotypes associated with HIV-SN

FastPHASE, applied to each gene separately, yielded 33 haplotypes which occurred at greater than 1% in this population (Table 2). Of these, 14 haplotypes were from *P2X7R* and accounted for 88% of patients, 5 in *P2X4R* accounted for 97% and 14 in *CAMKK2* accounted for 89%. Each haplotype is presented using the individual SNP alleles [1(major) or 2 (minor)] as determined in this population. These are ordered to match their chromosomal position (as in Table 1).

No haplotypes significantly associated with HIV-SN but three in *P2X7R*, one in *P2X4R* and four in *CAMKK2* met the criterion ($p < 0.2$) for inclusion in logistic regression modelling (Table 2). The optimal model linked viremia and a lower current CD4 T cell count, plus *CAMKK2* haplotypes 6 and 11 and *P2X7R* haplotypes 2 and 12 with HIV-SN (model $p = 0.0002$; pseudo $R^2 = 0.11$; Table 3). Haplotypes 6 and 11 lack rs1560568*A, rs7975295*C or rs1132780*A (which associated with protection). The associations of *P2X7R* haplotypes 2 and 12 with HIV-SN were in the opposite direction in the optimal model and neither associated with HIV-SN in bivariate analyses.

CAMKK2 haplogroups associated with reduced risk of HIV-SN

Haplotype networks were constructed for *P2X7R*, *P2X4R* and *CAMKK2* individually, but no distinct haplogroups were identified in *P2X7R* and *P2X4R* (Online Resource 2 and 3).

The 64 *CAMKK2* haplotypes segregated into four haplogroups (denoted A-D; Fig. 1.). This accounted for 27 of the 64 haplotypes and 80% of the patient population. Haplogroups C and D contained two and three haplotypes, and each accounted for only 1% of this population so only haplogroups A and B were assessed for associations with HIV-SN.

CAMKK2 haplogroup A included 16 haplotypes. Haplotypes from within this group were carried by 49% (17/35) of patients with HIV-SN and 69% (115/167) of patients without and associated with being free of HIV-SN ($p = 0.02$, OR = 0.43 CI = 0.21–0.88). Haplogroup A included two haplotypes which were not seen here in any individual with HIV-SN. All haplotypes in the group included the three alleles associated with lower rates of HIV-SN here [rs7975295*C, rs1560568*A, rs1132780*T], and/or rs3794204*G. rs3794204*G is an intronic SNP which showed no association with HIV-SN on bivariate analyses but is carried in all haplotypes containing the three alleles of interest. Our findings support a protective role for the three shared alleles.

CAMKK2 haplogroup B covered six haplotypes and was carried by 20% (7/35) and 11% (19/167) of patients with and without HIV-SN, creating a weak association with HIV-SN ($p = 0.16$, OR = 1.95, CI = 0.76–5.08). Haplogroup B included *CAMKK2* haplotype 4 which also weakly associated with risk of HIV-SN ($p = 0.055$) and contains four minor alleles (rs1718158*G, rs1653586*T, rs1653587*G and rs3794204*G) but these did not associate with HIV-SN.

ANAPC5 genotypes did not associate with HIV-SN

DNA samples were genotyped for 10 SNPs in *ANAPC5* and 12 haplotypes which occurred at greater than 1% were derived. However, no SNPs or haplotypes associated with HIV-SN ($p = 0.2–1.0$; data not shown), so LD with *ANAPC5* does not explain the protective effect of rs7975295*C, rs1560568*A and/or rs1132780*T in *CAMKK2*.

Discussion

HIV-SN is a neurological complication which remains clinically important even though recommended antiretroviral regimens no longer include neurotoxic drugs such as stavudine. This study investigated genotypes of *P2X7R*, *P2X4R* and *CAMKK2* that were associated with HIV-SN in an African population treated with stavudine (Goullee et al. 2016). Here, we describe a cohort of Indonesian HIV patients who had never received stavudine. HIV-SN was associated with CD4 T cell counts and HIV RNA. This contrasted with the association with age and height seen in the same clinic population 10 years earlier when patients received stavudine (Octaviana et al. 2018) and establishes the possibility that genetic associations may illuminate distinct mechanisms underlying HIV-SN in patients who do not receive stavudine. Here, our optimal model considering SNPs included a lower CD4 T cell count and viremia at the time of assessment and rs1560568*A in *CAMKK2*. This allele is co-inherited with rs7975295*C and rs1132780*T, so either of these or a linked (untyped) SNP may exert the protective effect. Similarly, the optimal model considering haplotypes included a lower CD4 T cell count and viremia at the time of assessment plus four haplotypes (*P2X7R*-2, *P2X7R*-12, *CAMKK2*-2 and *CAMKK2*-11).

As the genes lie in a region of high LD and within a TAD, it is necessary to consider whether these associations reflect the functions of P2X4R, P2X7R or CaMKK2. The *P2X7R*-2 and *P2X7R*-12 (2211211211121 and 2211111211121) haplotypes had opposing associations with HIV-SN in the optimal model (OR = 0.44 versus OR = 11.37; Table 3). These haplotypes differ by one allele, rs1186055*T, an intronic variant which was associated with risk of HIV-SN in South African HIV+ patients (Goullee et al. 2016) but not in this study (Table 1). These two haplotypes also shared four minor alleles (rs10849849*G, rs1718125*A, rs7132846*T and rs3751142*A). Rs1718125*A was associated with sensitivity

Table 2 Eight haplotypes from *P2X7R*, *P2X4R* and *CAMKK2* weakly associated with HIV-SN

Haplotype Number	Haplotype Alleles	Without HIV-SN[a]		With HIV-SN[b]		P (Chi2)[c]
P2X7R						
1	1121111111111	102	61%	21	60%	0.91
2	2211211211121	52	31%	6	17%	0.10
3	2211211111111	22	13%	6	17%	0.54
4	1211122121112	24	14%	4	11%	0.79
5	1111222121112	13	8%	2	6%	1.00
6	1121211111111	8	5%	1	3%	1.00
7	2211111111111	9	5%	1	3%	1.00
8	1121211211121	8	5%	3	9%	0.41
9	1211111111111	11	7%	0	0%	0.22
10	1121111211121	4	2%	3	9%	0.10
11	2211112121112	8	5%	2	6%	0.69
12	2211111211121	2	1%	2	6%	0.14
13	1211121111111	6	4%	0	0%	0.59
14	1111111111111	2	1%	0	0%	1.00
P2X4R						
1	1111111	132	79%	27	77%	0.80
2	2222111	87	52%	14	40%	0.19
3	2212111	24	14%	8	23%	0.21
4	1112111	8	5%	2	6%	0.69
5	2112111	3	2%	1	3%	0.54
CAMKK2						
1	121111221221111111	85	51%	13	37%	0.14
2	111112112111111111	53	32%	15	43%	0.21
3	111121111111111111	47	28%	8	23%	0.52
4	212211211111111111	15	9%	6	17%	0.15
5	111121111111112211	14	8%	4	11%	0.57
6	111121111111112212	16	10%	6	17%	0.19
7	111121221221111111	12	7%	1	3%	0.47
8	111111211111111111	6	4%	2	6%	0.63
9	111121111111111112	5	3%	2	6%	0.35
10	121111221221111112	4	2%	0	0%	1.00
11	111112112111111112	2	1%	2	6%	0.14
12	121111221222122211	4	2%	0	0%	1.00
13	111112112111112212	5	3%	0	0%	0.59
14	212111211111111111	3	2%	1	3%	0.54

a. Number of individuals without HIV-SN who carry 1 or 2 copies of the haplotype
b. Number of individuals with HIV-SN who carry 1 or 2 copies of the haplotype
c. Fisher's Exact was used where n<5
d. Eight haplotypes which met the criteria for inclusion in logistic regression modelling (p<0.2) are shaded

to cold pain (Ide et al. 2014) and is found in a haplotype independently associated with HIV-SN in South African HIV+ patients receiving stavudine (Goullee et al. 2016), as was rs10849849*G. As all four shared SNPs are in non-

Table 3 Optimal logistic regression models show *CAMKK2* SNPs and haplotypes independently associate with HIV-SN

Variable	Odds ratio	*P* value	95% confidence interval
Optimal model including demographic and clinical variables and SNPs			
$n = 195$[a], $p = 0.0002$, pseudo $R^2 = 0.11$			
Current CD4 T cell Count	1.00	0.009	0.99–1.00
Current Viremia	3.55	0.047	1.02–12.40
rs7975295 / rs1560568 / rs1132780 (*CAMKK2*)	0.43	0.038	0.20–0.95
Optimal model including demographic and clinical variables and haplotypes			
$n = 195$[a], $p = 0.0002$, pseudo $R^2 = 0.11$			
Current CD4 T cell count	1.00	0.003	0.99–1.00
Current viremia	4.62	0.020	1.28–16.7
P2X7R-2: 2211211211121	0.44	0.128	0.16–1.26
P2X7R-12: 2211111211121	11.37	0.023	1.39–92.8
CAMKK2–6: 11112111111112212	2.21	0.158	0.73–6.65
CAMKK2–11: 111112112111111112	18.98	0.008	2.13–169

[a] Excluding samples with missing demographic, clinical and/or genotyping data

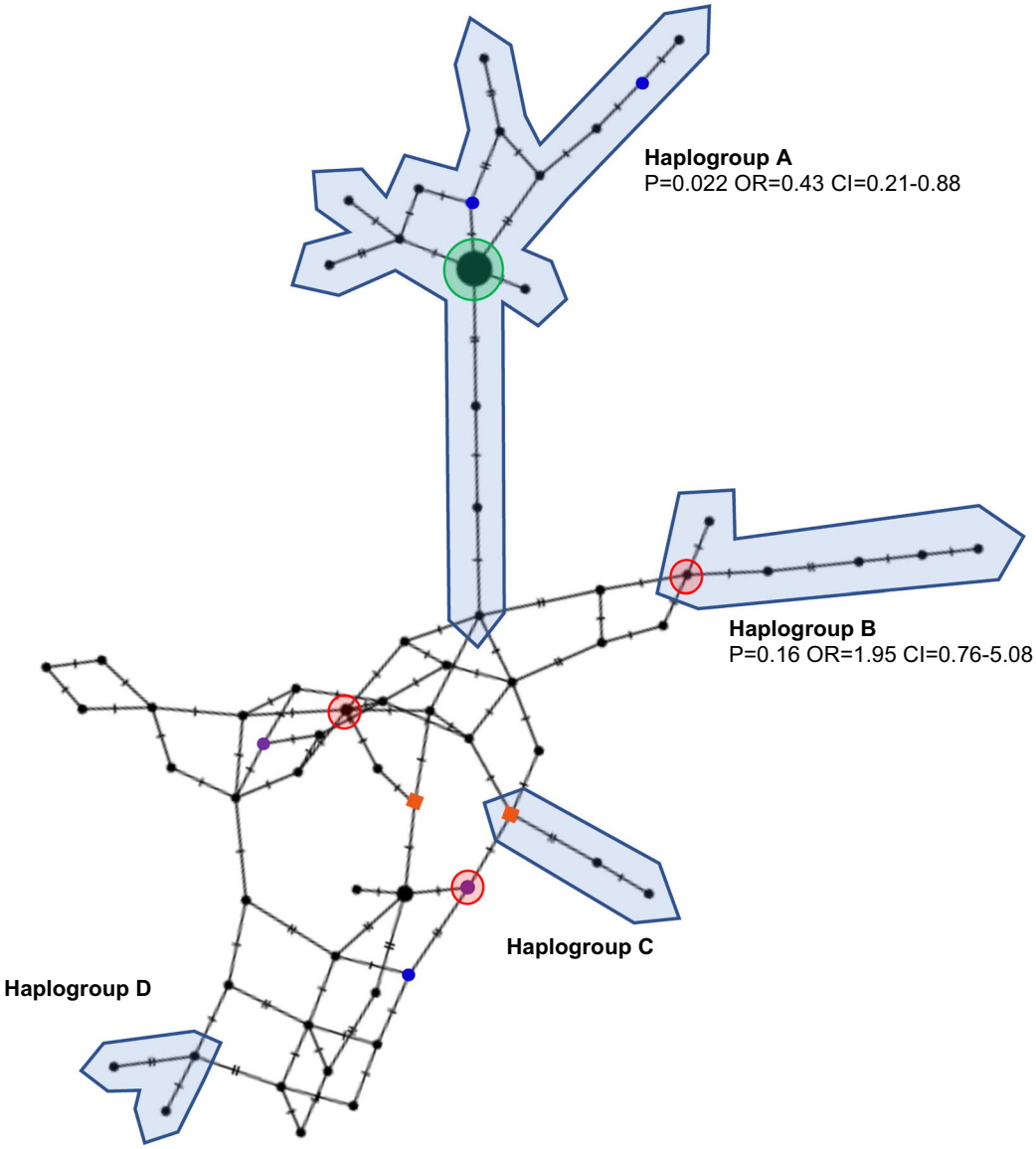
coding regions, we considered evidence that they may influence expression of *P2X7R* or neighbouring genes. Accordingly, the GTEX eQTL database links carriage of the minor allele of rs7132846*T and rs3751142*A with increased expression of P2X4R in many cell types, including tibial nerves (https://gtexportal.org/home/). This is consistent with a role for P2X4R in the manifestation of HIV-SN despite the lack of association with SNPs in that gene.

However, several lines of evidence also support a role for CaMKK2:

1. *CAMKK2* haplotype 6 included the minor alleles rs2686367*C, rs11065504*C, rs3817190*A and rs9805130*A where the latter two are in complete LD. The rs3817190*A allele changes a threonine to serine at the 85th amino acid in exon 1. This variant has been associated with severity of panic and agoraphobia scores in patients with anxiety disorders and reduces autonomous activity of CaMKK2 (Barden et al. 2006; Erhardt et al. 2007; Scott et al. 2015). However, as this SNP was not associated on univariate analyses ($p = 0.99$), it is unlikely to be causative.
2. *CAMKK2* haplotype 11 included the minor alleles rs2686367*C, rs2686342*T and rs2686344*T. Carriage of these three alleles is associated with increased expression of CaMKK2 (https://gtexportal.org), a characteristic typically associated with disease phenotypes and poorer outcomes.
3. Three polymorphisms in *CAMKK2*, rs7975295*C, rs1560568*A and rs1132780*T, associated with reduced risk of HIV-SN in bivariate analyses ($p < 0.05$; OR = 0.44–0.46; 95% CI = 0.21–0.99; Table 1), were retained in the optimal model predicting HIV-SN ($p = 0.0002$, pseudo $R^2 = 0.11$; Table 3). We also identified a haplogroup marked by carriage of all three minor alleles which associated with reduced risk ($p = 0.022$; Fig. 1.)

Here, we discuss these three SNPs and mechanisms by which they may impact HIV-SN. rs7975295*C and rs1560568*A independently associated with increased risk of HIV-SN but were found within haplotypes which associated with reduced risk of HIV-SN in South African HIV+ patients (Goullee et al. 2016). This suggests the pattern of LD in South Africans may differ from Indonesians. The two SNPs lie in non-coding regions. The GTEX eQTL database links carriage of both minor alleles with increased expression of P2X4R in many tissues, notably tibial nerves, consistent with a role in disease progression. However, only a single non-synonymous *P2X4R* SNP, rs25644*G, weakly associated with reduced risk of HIV-SN ($p = 0.14$, OR = 0.58, CI95% = 0.28–1.24; Table 1) and this did not remain in the optimal model. Investigations of the impact of rs7975295*C and rs1560568*A on the regulation of CaMKK2 are warranted.

Rs1132780 is located in a coding region and carriage of the T (minor) allele results in a non-synonymous change of arginine to cysteine at position 363 in the kinase domain. The SIFT web server predicts this change to be deleterious in several isoforms (see Online resource 4) and so may contribute to HIV-SN via altered activity of CaMKK2 and/or its substrates. Altered CaMKK2-dependent activity of AMPK, SIRT1, CAMKI and CAMKIV has been implicated in peripheral neuropathies and neuropathic pain. For example, Calcutt et al. (2017) demonstrated that inhibition of CaMKK2-dependent AMPK activity with antimuscarinic drugs prevented or reversed thermal hypoalgesia and reduced intraepidermal nerve fibre density in diabetic, chemotherapy-induced and HIV-gp120-induced peripheral neuropathies in rodents. Additionally, Shao et al. (2014) demonstrated that



Symbol	Definition
■	Median vector: hypothetical haplotype automatically generated for maximum parsimony
●	Haplotypes contained within the optimal statistical model
●	Haplotypes not seen in patients with HIV-SN
○	Haplotypes weakly associated (p<0.2) with risk of HIV-SN in bivariate analyses
○	Haplotypes weakly associated (p<0.2) with protection of HIV-SN in bivariate analyses
●	Node size is proportional to haplotype frequency in this population

Fig. 1 *CAMKK2* haplogroup A associated with reduced risk of HIV-SN

stimulation of SIRT1 ameliorated neuropathic pain in rodent models. Altered CaMKI and CaMKIV activity is also implicated in peripheral neuropathies and neuropathic pain (Elzière et al. 2014; Zhao et al. 2016). The structural and functional impact of rs1132780*T should be investigated.

Overall, we have identified three candidate SNPs in *CAMKK2* which are co-inherited and associated with reduced prevalence of HIV-SN in Indonesian patients who have never received stavudine. Whilst we acknowledge the small size of the cohort, preliminary screening of patients eliminated unrelated causes of neuropathy. Our approach based on multivariable analyses identifies the strongest determinants of risk, which then warrant validation in an independent cohort. The findings provide new insight into possible mechanisms of HIV-SN independent of those initiated by stavudine. Future studies should explore the functional impact of carriage of rs1132780*T and regulatory roles of rs7975295*C and rs1560568*A in the context of HIV-SN. Finally, genetic studies should consider interactions between the *P2X*-block and the remaining 26 genes within this TAD (Online Resource 1). We are now addressing ethnic differences and genotypes affecting large fibre damage in this cohort.

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Compliance with ethical standards

The study was approved by the Ethics Committee of the Faculty of Medicine, Universitas Indonesia. Written and informed consent was obtained.

Conflict of interest The authors declare that they have no conflict of interest.

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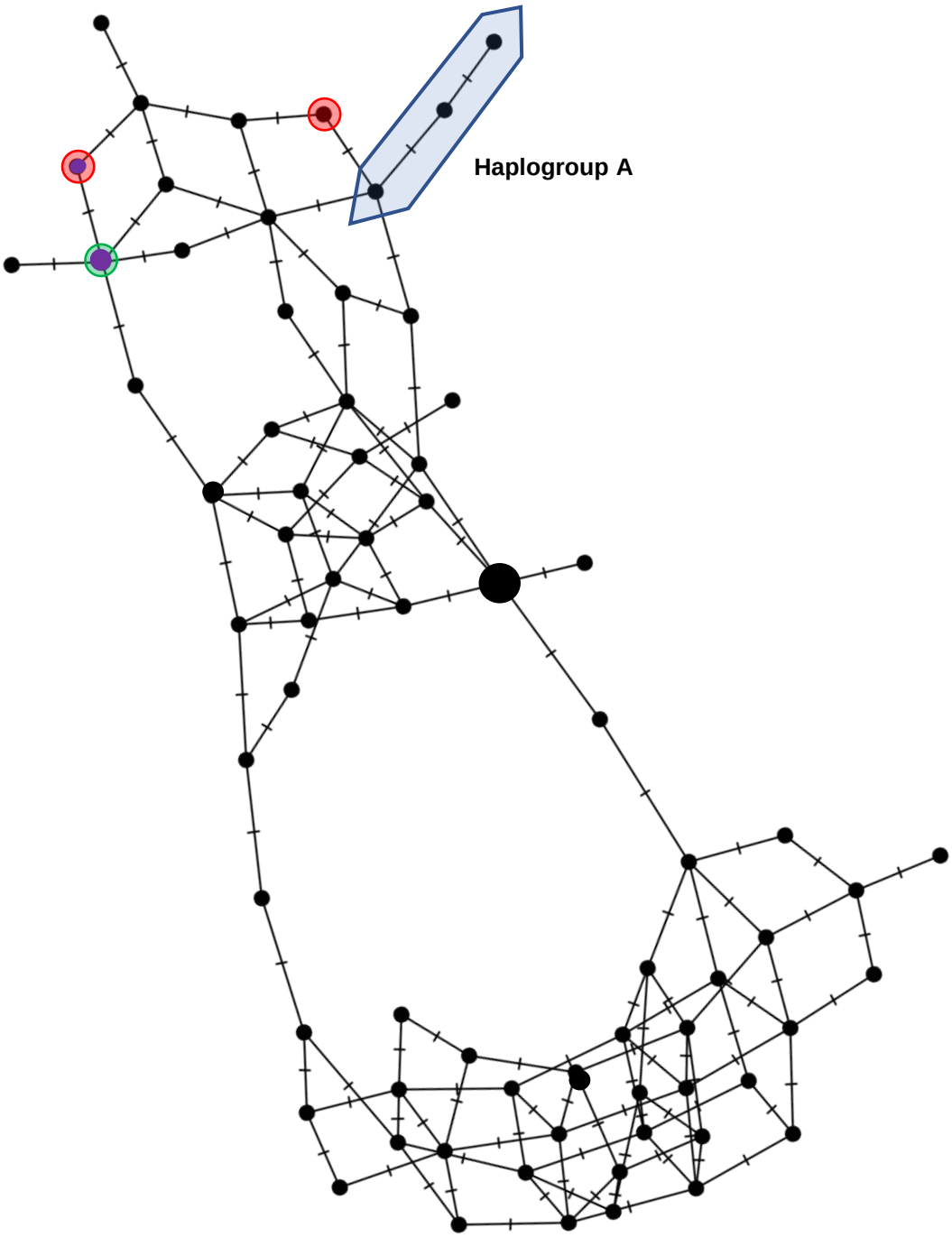
Online Resource 1. The larger TAD in astrocytes from the cerebellum and spinal cord spans chromosome 12: 120600001-121840000bp and contains 30 genes.

Location (Chromosome 12)[a]	Gene ID	Gene Name
120565014 – 120632513	*GCN1*	GCN1, eIF2 alpha kinase activator homolog
120634502 – 120639014	*RPLPO*	Ribosomal protein lateral stalk subunit P0
120639094 – 120650631	*PXN-AS1*	PXN antisense RNA 1
120648242 – 120703574	*PXN*	Paxillin
120730002 – 120751045	*SIRT4*	Sirtuin 4
120759914 – 120765592	*PLA2G1B*	Phospholipase A2 group IB
120779133 – 120806983	*MSI1*	Musashi RNA binding protein 1
120875893 – 120878545	*COX6A1*	Cytochrome c oxidase subunit 6A1
120881764 – 120884215	*TRIAP1*	TP53 regulated inhibitor of apoptosis 1
120884241 – 120901556	*GATC*	Glutamyl-tRNA amidotransferase subunit C
120899471 – 120907558	*SRSF9*	Serine and arginine rich splicing factor 9
120907660 – 120936298	*DYNLL1*	Dynein light chain LC8-type 1
120928141 – 120933749	*NRAV*	Negative regulator of antiviral response
120941082 – 120966964	*COQ5*	Coenzyme Q5, methyltransferase
120972132 – 121015397	*RNF10*	Ring finger protein 10
121016848 – 121019201	*POP5*	POP5 homolog, ribonuclease P/MRP subunit
121078422 – 121105129	*CABP1*	Calcium binding protein 1
121124949 – 121139667	*MLEC*	Malectin
121148238 – 121161443	*Unc119b*	Unc-119 lipid binding chaperone B
121160996 – 121161069	*Mir4700*	MicroRNA 4700
121163544 – 121177811	*ACADS*	Acyl-CoA dehydrogenase short chain
121200313 – 121342155	*SPPL3*	Signal peptide peptidase like 3
121407641 – 121410095	*HNF1A-AS1*	HNF1A antisense RNA 1
121415861 – 121440315	*HNF1A*	HNF1 homeobox A
121440835 – 121454300	*C12orf43*	Chromosome 12 open reading frame 43
121458095 – 121477045	*OASL*	2'-5'-oligoadenylate synthetase like
121570622 – 121624439	***P2X7R***	**Purinergic receptor P2X 7**
121647664 – 121671909	***P2X4R***	**Purinergic receptor P2X 4**
121675495 – 121736111	***CAMKK2***	**Calcium/calmodulin dependent protein kinase kinase 2**
121746048 – 121792012	***ANAPC5***	**Anaphase promoting complex subunit 5**

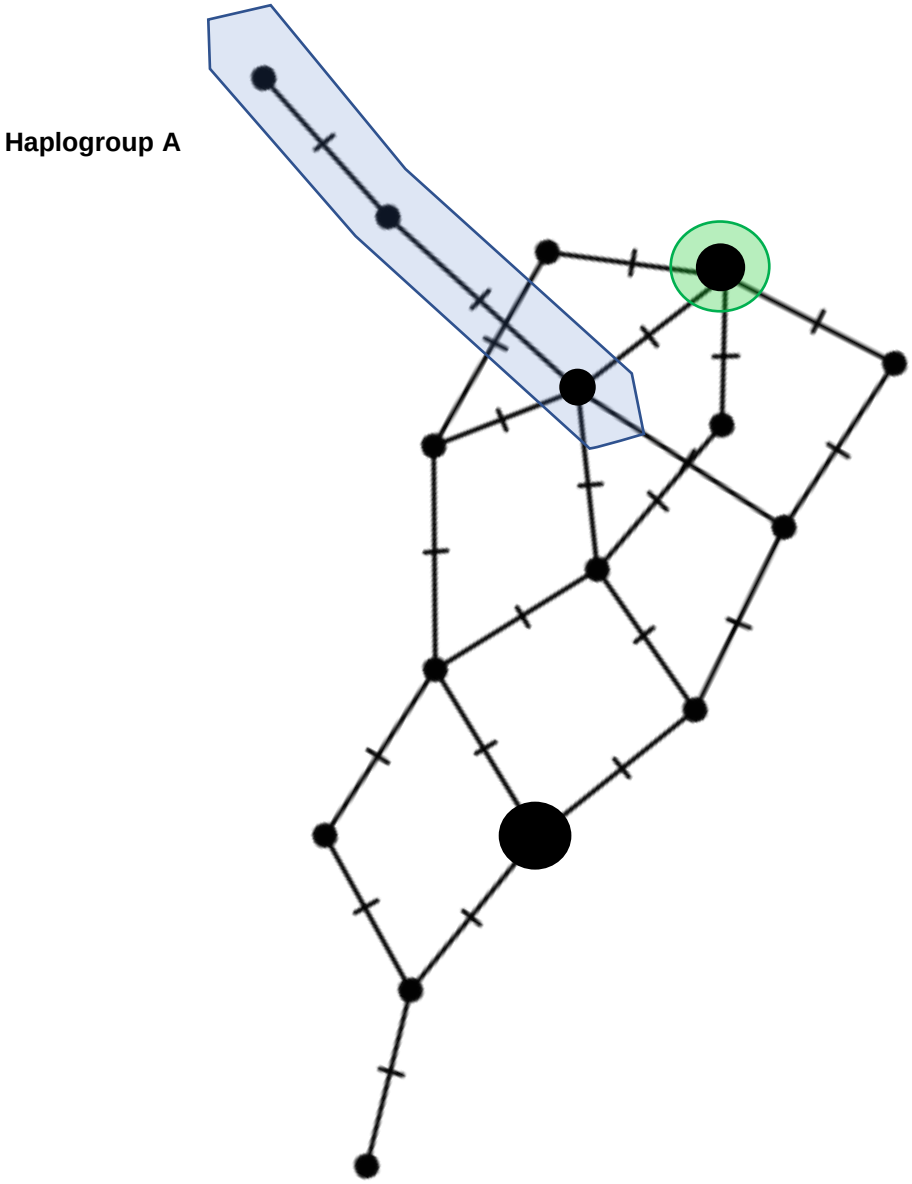
[a] Genome Assembly GRCh37
[b] Genes assessed in this study are in bold

Online Resource 2. No distinct *P2X7R* haplogroups were identified



Symbol	Definition
	Median vector: hypothetical haplotype automatically generated for maximum parsimony
	Haplotypes contained within the optimal statistical model
	Haplotypes not seen in patients with HIV-SN
	Haplotypes weakly associated (p<0.2) with risk of HIV-SN in bivariate analyses
	Haplotypes weakly associated (p<0.2) with protection of HIV-SN in bivariate analyses
	Node size is proportional to haplotype frequency in this population

Online Resource 3. No distinct *P2X4R* haplogroups were identified



Symbol	Definition
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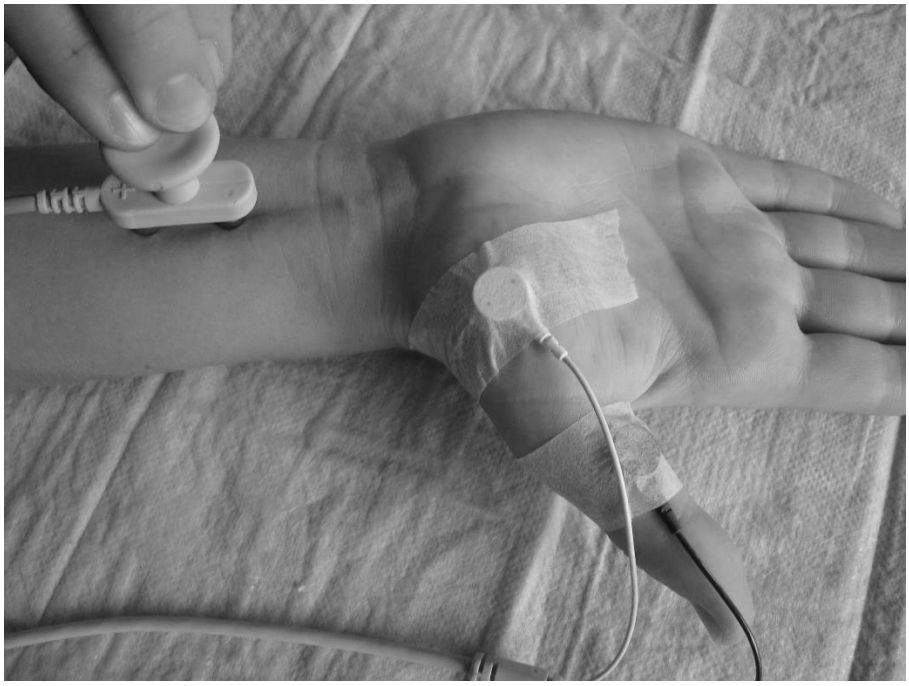
Chapter 5

Demographic and genetic associations with markers of small and large fiber sensory neuropathy in HIV patients treated without stavudine

Genetic investigation of the P2X-block in Indonesians and Africans treated without stavudine identified a clear link between HIV-SN and CAMKK2. Given the role of the protein encoded by CAMKK2 in neuronal growth and repair, I assessed whether this link may reflect the small or large nerve fibre pathology in HIV-SN. In Chapter 5 I compare demographic, clinical and P2X-block genetic risk factors of large and small fibre neuropathy with HIV-SN in a subset of the Indonesian cohort.



Data from this chapter have been published:

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Demographic and Genetic Associations With Markers of Small and Large Fiber Sensory Neuropathy in HIV Patients Treated Without Stavudine

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Abstract: Neurotoxic antiretroviral therapy (ART) such as stavudine has been now replaced with safer therapies, reducing the prevalence of neuropathy from 34% to 15% in HIV+ Indonesians. However, it is unclear whether the residual cases display damage to small or large nerve fibers and whether both are influenced by known risk factors, including alleles of *CAMKK2* associated with neuropathy in HIV patients. The encoded protein influences the growth and repair of nerve fibers. HIV-positive adults on ART for >12 months without exposure to stavudine were screened for neuropathy using the AIDS Clinical Trials Group Brief Peripheral Neuropathy Screen (BPNS). Large fiber neuropathy was assessed by nerve conduction (NC) and small fiber neuropathy using stimulated skin wrinkling (SSW) applied to the fingers. *CAMKK2* alleles were assessed by TaqMan OpenArray technology. Neuropathy diagnoses were more common with SSW than BPNS (49/173 vs 26/185, χ^2; $P = 0.0009$), with poor alignment between these outcomes ($P = 0.60$). NC and BPNS diagnosed neuropathy at similar frequencies (29/151 vs 26/185; $P = 0.12$) and were aligned ($P < 0.0001$). In bivariate analyses, all diagnoses were associated with patients' age and persistent HIV replication, with minor effects from CD4 T-cell counts and time on ART. *CAMKK2* alleles associated with neuropathy diagnosed with BPNS and SSW but not NC. Multivariable analyses confirmed the importance of age and HIV replication, with distinct *CAMKK2* polymorphisms affecting BPNS and SSW. Paradoxically, height was protective against skin wrinkling. Overall the data link *CAMKK2* genotypes with small rather than large fiber damage. SSW may reflect pathology distinct from that identified using BPNS.

Key Words: HIV, sensory neuropathy, risk factors

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INTRODUCTION

Management of people living with HIV (PLWH) can now focus on their quality of life as antiretroviral therapy (ART) increases life expectancy. However, HIV-associated sensory neuropathy (HIV-SN) still arises, and debate remains regarding the roles of HIV infection itself and different modalities of ART. Neurotoxic ART such as stavudine has been now replaced with safer therapies, reducing the prevalence of HIV-SN from 34% to 15% in Indonesian PLWH,[1] but the condition remains problematic. Patients describe unusual sensations, such as neuropathic pain, tingling, numbness, hyperalgesia, and allodynia. Physical examination may reveal nonspecific signs including loss of ankle reflexes and sensory loss in the distal part of the feet.[2,3] An established tool validated to diagnose HIV-SN is the AIDS Clinical Trial Group Brief Peripheral Neuropathy Screen (BPNS). HIV-SN is diagnosed if there is at least one listed symptom, plus decreased Achilles reflexes or decreased sensibility to vibration when a tuning fork is held on a toe.[4] These tests detect small and large fiber neuropathy, and it remains unclear which risk factors affect small and large nerves.

Nerve conduction (NC) studies assess sensory and motor conduction of an electrical impulse to identify sensory motor deficits that affect large fiber nerves[5] and may trigger weakness, loss of joint position and vibration sense, and sensory ataxia. Small fiber neuropathy manifests as neuropathic pain, impairment of temperature, and autonomic function and is more difficult to assess. Intraepidermal nerve fiber density is considered to be the optimal criterion to detect damage to small diameter sensory nerves, including non-myelinated and myelinated intraepidermal nerve fibers. The European Federation of Neurological Societies recommends a biopsy of skin to a depth of 3 mm by using a skin punch biopsy on the distal limbs to calculate the linear density or nerve fibers with a minimum of 50 μm thickness slices. Nerve fibers can be stained with antibodies recognizing PGP9.5 and visualized using confocal microscopy,[6] but the technique is invasive and unsuitable for routine screening. Stimulated skin

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wrinkling (SSW) can assess small nerve fiber function using exposure to eutectic mixture of local anesthetics. Skin wrinkling occurs as result of vasoconstriction in the glabrous skin, mediated by postganglionic sympathetic fibers.[7] SSW has been correlated with intraepidermal nerve fiber density in patients with sensory neuropathy.[8] The sensitivity of SSW test for diagnosing diabetic sensorimotor polyneuropathy using NCS as a reference standard was 81.3%, and specificity was 67.0% The authors noted that this was comparable with other testing methods, but it is difficult to interpret as NC assesses large nerve fibers.[9]

Our genetic studies identified a role for polymorphisms in the *CAMKK2* gene in HIV-SN diagnosed using BPNS in South African PLWH receiving stavudine.[10] *CAMKK2* polymorphisms and haplotypes aligned with BPNS positivity were different in Indonesian and African patients treated without stavudine, but polymorphisms in this gene were retained in the optimal multivariable models for each cohort.[11,12] The encoded protein, CaMKK2 (calcium/calmodulin kinase 2) phosphorylates AMPK (AMP-activated protein kinase), SIRT1 (sirtuin 1), CaMKIV, and CaMKI (calcium/calmodulin kinase 4 and 5).[13,14] SIRT1 regulates axonal regeneration, protects neurons from oxidative stress, and promotes dendrite arborisation.[15] CaMKIV activation upregulates NFκB and cAMP (cyclic adenosine monophosphate) response element-binding protein which stimulates brain-derived neurotrophic factor promoting neuronal growth and survival.[14] Brain-derived neurotrophic factor has been implicated in neuropathic pain.[16] Activated CaMKI regulates axonal growth cone morphology and outgrowth, dendrite arborisation, and synapse formation.[17] A possible explanation for associations with different *CAMKK2* genotypes is a shift in the relative importance of small and large fibers in the pathogenesis of HIV-SN in different populations and with the cessation of stavudine. This is addressed here in Indonesian PLWH assessed by BPNS, NC, and SSW. Associations with genetic and demographic factors are compared.

METHODS

HIV-positive adults who had used ART for at least 12 months without exposure to stavudine were screened for neuropathy at POKDISUS HIV Care Clinic, Cipto Mangunkusumo Hospital, Jakarta, Indonesia.[1] Laboratory, clinical, and demographic data were collected from the medical files. Plasma HIV RNA was measured using a Cobas Amplicor Monitor (Roche Molecular Diagnostics, Pleasanton, CA). The study was approved by the Ethics Committee of the Faculty of Medicine, University of Indonesia (579/UN2.F1/ETIK/2014). All participants gave written informed consent.

Neuropathy was assessed using the AIDS Clinical Trial Group-BPNS and defined as present if the individual had one or more of the lower limb neuropathic symptoms (pain, aching or burning, pins and needles, or numbness), plus absent ankle reflexes, or reduced vibration sense at the great toe (vibration of a 128-Hz tuning fork felt for 10 seconds or less). NC was assessed according to protocols of the American Association of Neuromuscular and Electro Diagnostic Medicine and compared with normative values from our clinic. Motor and sensory NC was assessed bilaterally in lower limbs for tibial, peroneal, and sural nerves and in upper limbs for median and ulnar nerves. NC positivity was defined as decrease of sensory nerve action potential amplitude (microvolt) $<$ 80% lower limit of normal values or absent in 2 or more nerves in different extremities.[18] To assess SSW, the distal digit pulp of the left second, third, and fourth fingers was covered with 5% EMLA cream (Eutectic Mixture of Local Anaesthetics; lidocaine 2.5% and prilocaine 2.5%; AstraZeneca, Cambridge, United Kingdom) for 30 minutes. Wrinkling was graded using a published scale from 0 (no wrinkling) to 4 (normal wrinkles).[8] Results from the 3 digits were added, and individuals scoring less than 9 were defined as SSW (+).

DNA extracted from EDTA-blood samples using FavorPrep Blood Genomic DNA Extraction Mini Kit (Favorgen Biotech Corporation, Changzhi, Taiwan) was adjusted to 50 ng/μL, diluted 1:1 with TaqMan OpenArray Genotyping Master Mix and genotyped for single nucleotide polymorphisms (SNPs) spanning *CAMKK2* using custom TaqMan OpenArray Real-Time polymerase chain reaction Plates using the QuantStudio 12K Flex Real-Time polymerase chain reaction System (Life Technologies, Grand Island, NY). Genotypes were assigned manually using TaqMan Genotyper Software. All SNPs conformed with Hardy–Weinberg Equilibrium, excepting 2 omitted because they were monoallelic in Indonesians (rs11837114 and rs7965129).[11]

Demographic, clinical, and treatment details of patients with and without neuropathy were compared using χ^2 tests (dichotomous variables) or Mann–Whitney tests [non-normally distributed continuous variables, described using median (range)], using GraphPad Prism version 8.2.1 for Windows (GraphPad Software, La Jolla, CA) without corrections for multiple comparisons. Multivariable analyses of associations with neuropathy were performed using multiple logistic regression modeling, undertaken using Stata/IC 16.0 for Windows (StataCorp LLC, College Station, TX). Modeling including all factors with $P < 0.20$ in bivariate analyses, followed by a stepwise removal process. Odds ratios and 95% confidence intervals are presented.

RESULTS AND DISCUSSION

Age and Persistent HIV Replication Promote Small and Large Fiber Neuropathy

After screening 2596 patients, 2411 were excluded for reasons including $<$12 months continuously on ART, past/current stavudine, diabetes mellitus, stroke, schizophrenia, vasculitis, deafness, blindness, hyperthyroidism, systemic lupus erythematous, cytomegalovirus radiculopathy, and cancer chemotherapy.[1] Demographic and clinical characteristics of 185 patients are described in Table 1. Neuropathy diagnoses were more common with neuropathy diagnosed with SSW than BPNS (χ^2; $P = 0.0009$), with poor alignment between these outcomes (6/49 vs 19/124; $P = 0.65$). This was investigated further using aspects of the BPNS believed to assess damage to small fibers. Patients positive and negative

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TABLE 1. Demographic and Genetic Factors Align Differentially With BPNS, Nerve Conduction, and Stimulated Skin Wrinkling

	BPNS (+) n = 26	BPNS (−) n = 159	*P*	NC (+) n = 29	NC (−) n = 122	*P*	SSW (+) n = 49	SSW (−) n = 124	*P*
Male	15	103	0.51	24	88	0.25	32	73	0.49
Age, yr	37 (29–59)	35 (22–60)	**0.17**	36 (22–59)	35 (23–60)	**0.08**	36 (23–60)	35 (22–50)	***0.04***
Height, cm	167 (151–175)	166 (142–180)	0.89	167 (153–178)	165 (142–180)	0.50	165 (142–178)	167 (148–180)	***0.02***
>500 HIV RNA copies/mL	4	6	**0.03**	5	4	***0.01***	7	3	***0.006***
Nadir CD4 T-cells/μL	65 (11–428)	122 (2–599)	0.29	86 (2–421)	130 (4–599)	**0.17**	90 (2–402)	123 (3–454)	0.40
Current CD4 T-cells/μL	397 (103–729)	457 (84–1166)	**0.09**	416 (103–795)	462 (84–1166)	0.30	433 (103–757)	465 (84–1166)	**0.19**
ART duration, yr	5.2 (1.0–10.9)	4.2 (1.0–12.7)	0.55	4.9 (1.1–12.2)	4.4 (1.0–12.7)	**0.14**	5.5 (1.2–12.2)	4.0 (1.0–12.7)	**0.10**
History of tuberculosis	15	64	**0.07**	14	48	0.38	21	51	0.84
Carriage of the minor allele of SNP in CAMKK2 (minor/major allele)									
rs1718158 G/A	23%	16%	0.35	10%	20%	0.29	17%	18%	0.87
rs10849861 A/G	40%	55%	**0.10**	55%	53%	0.83	49%	55%	0.45
rs1653586 T/G	23%	16%	0.35	10%	20%	0.29	17%	18%	0.87
rs1653587 G/A	20%	14%	0.35	7%	16%	**0.19**	14%	15%	0.86
rs11065504 C/G	46%	51%	0.58	54%	49%	0.68	61%	45%	***0.05***
rs2686342 T/A	49%	40%	0.34	41%	40%	0.93	37%	44%	0.39
rs3794204 G/A	63%	72%	0.27	69%	70%	0.89	71%	71%	1.00
rs7975295 C/T	40%	60%	***0.03***	66%	55%	0.32	59%	56%	0.71
rs2686344 T/C	49%	39%	0.27	41%	39%	0.80	35%	43%	0.31
rs1560568 A/G	40%	60%	***0.03***	66%	56%	0.34	59%	56%	0.74
rs1132780 T/C	40%	61%	***0.04***	66%	56%	0.34	60%	56%	0.61
rs7314454 T/C	6%	9%	0.74	3%	8%	0.69	13%	6%	**0.13**
rs1109453 A/G	9%	13%	0.77	7%	12%	0.74	13%	11%	0.72
rs3817190 A/T	34%	34%	0.99	38%	32%	0.54	43%	27%	***0.05***
rs9805130 A/G	34%	35%	0.94	38%	34%	0.66	47%	27%	***0.01***
rs2686367 C/A	29%	23%	0.51	34%	23%	**0.18**	26%	23%	0.77

Variables achieving $P < 0.20$ are bolded.
Variables achieving $P < 0.05$ are bold and italics.

for neuropathy diagnosed with SSW (resp.) displayed similar frequencies of neuropathic pain (5/49 vs 7/124; $P = 0.29$), tingling in the feet (4/49 vs 9/124, $P = 0.84$), and numbness in their feet (5/49 vs 12/124, $P = 0.92$). Hence, SSW is a poor marker of neuropathy captured by BPNS or its components.

NC and BPNS positivity occurred at similar frequencies ($P = 0.12$) and were significantly aligned ($P < 0.0001$). NC assessments (including distal latency, amplitude and morphology of CMAP/SNAP, and conduction velocity) can distinguish demyelination from axonal degeneration. Changes observed in this cohort were consistent with axonal degeneration. As described previously,[1] factors weakly associated with BPNS positivity ($P < 0.2$) include age, >500 copies HIV RNA/mL, current CD4 T-cell counts, and a history of tuberculosis. We also associated NC positivity with slightly greater age and time on ART ($P < 0.2$) with a clear effect of >500 copies HIV RNA/mL ($P = 0.01$). When demographic factors achieving $P < 0.2$ were assessed in multivariable analyses, BPNS and NC positivity were independently associated with greater age and HIV replication (Table 2).

SSW positivity associated with slightly reduced height, increased age, and time on ART, and >500 copies HIV RNA/mL (Table 1). The optimal multivariable model for SSW included height as a protective factor. This was unexpected as height was a risk factor for neuropathy in patients receiving stavudine.[1] As a link between skin wrinkling (ie,: SSW negativity) and poor circulation in the fingers has been demonstrated,[7] we speculate that wrinkling may be accentuated in taller individuals, but this requires verification. We are aware that HIV-SN rarely affects the hands but dysfunction of the sympathetic fibers as shown by an abnormal SSW are likely to be systemic as they reflect sympathetic nerve function.[7]

SSW and BPNS Associate With Different Alleles of *CAMKK2*, but *CAMKK2* Does Not Affect NC

As described previously,[10] alleles of 3 polymorphisms in *CAMKK2* (rs7975295*C, rs1560568*A, and rs1132780*T) associated with reduced risk of HIV-SN assessed by BPNS in bivariate analyses ($P < 0.05$; odds ratio = 0.44–0.46; 95% confidence interval = 0.21 to 0.99; Table 1). As these alleles are in complete linkage disequilibrium, only rs1560568 was carried forward into multivariable analyses, along with rs10849861 (Table 2). rs1560568 was retained in the optimal model predicting BPNS positivity. rs1132780 is located in a coding region and carriage of the T (minor) allele changes an arginine to cysteine at position 363 in the kinase domain.[11] This should be investigated further.

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TABLE 2. Optimal Multivariable Models Predicting Small and Large Fiber Neuropathy Highlight Age and Persistent Viral Replication but are Differentially Improved by the Inclusion of *CAMKK2* Alleles

	OR	95% CI	*P*
Prediction of BPNS (demographics only): Pseudo $R^2 = 0.058$, $P = 0.034$, n = 185			
Age	1.062	0.99 to 1.14	0.078
>500 copies HIV RNA/mL	4.737	1.20 to 18.7	0.026
Prediction of BPNS (demographics & *CAMKK2* genotype): Pseudo $R^2 = 0.0747$, $P = 0.011$, n = 185			
Age	1.055	0.99 to 1.13	0.118
>500 copies HIV RNA/mL	5.175	1.27 to 20.9	0.021
rs1560568/rs7975295/rs1132780	0.422	0.17 to 1.02	0.056
Prediction of NC (demographics only): Pseudo $R^2 = 0.0702$, $P = 0.006$, n = 151			
Age	1.070	1.00 to 1.14	0.046
>500 copies HIV RNA/mL	7.177	1.73 to 29.8	0.007
Prediction of NC (demographics & *CAMKK2* genotype): Pseudo $R^2 = 0.0810$, $P = 0.008$, n = 151			
Age	1.063	1.00 to 1.14	0.080
>500 copies HIV RNA/mL	8.003	1.86 to 34.3	0.005
rs2686367	1.840	0.72 to 4.71	0.204
Prediction of SSW (demographics only): Pseudo $R^2 = 0.0868$, $P = 0.0005$, n = 173			
ART duration	1.108	0.99 to 1.23	0.063
Height	0.943	0.90 to 0.99	0.011
>500 copies HIV RNA/mL	6.767	1.60 to 28.6	0.009
Prediction of SSW (demographics & CAMKK2 genotype): Pseudo $R_2 = 0.1350$, $P = 0.0001$, n = 173			
ART duration	1.103	0.99 to 1.23	0.086
Height	0.936	0.89 to 0.98	0.007
>500 copies HIV RNA/mL	7.312	1.72 to 31.0	0.007
rs11065504	2.174	1.02 to 4.62	0.044
rs9805130	2.245	1.07 to 4.70	0.032

CI, confidence interval; OR, odds ratio.

NC positivity showed no significant associations with *CAMKK2* alleles, but rs1653587 and rs2686367 were carried forward into multivariable analyses. Table 2 shows a model retaining the most promising SNP (rs2686367) to illustrate the lack of any significant effect of *CAMKK2* genotypes. rs2686367 lies between *CAMKK2* and the adjacent gene *ANAPC5* (upstream of *CAMKK2*). From 10 SNPs in *ANAPC5*, an allele of rs74644631 associated with NC positivity (12% vs 29%, $P = 0.03$), as did 2 coinherited SNPs in the neighboring gene *KDM2B* (rs12427382, 63% vs 86%, $P = 0.02$; rs11065575 66% vs 89%, $P = 0.02$). *ANAPC5* encodes the anaphase promoting complex 5 (AnapC5), involved in progression from metaphase into anaphase. Replication of neurons would disrupt signal transduction so re-entry into the cell cycle triggers apoptosis.[19] Hence, altered function or expression of *ANAPC5* may promote neuronal death.

In contrast to BPNS and NC, bivariate analyses linked SSW positivity ($P < 0.20$) with alleles of rs11065504, rs7314454, rs3817190, and rs9805130 (Table 1), where the latter 2 were in linkage disequilibrium. Multivariable modeling of SSW positivity included rs11065504, rs7314454, and rs9805130. The optimal model retained significant independent associations with rs11065504 and rs9805130 genotypes (Table 2) and was stronger than the model obtained with demographic factors. The rs3817190*A allele changes a threonine to serine at the 85th amino acid in exon 1. This variant has been associated with panic and agoraphobia scores in patients with anxiety disorders and reduces autonomous activity of CaMKK2.[20] The findings reinforce the role of nerve damage in SSW.

CONCLUSIONS

We present a cohort study of PLWH attending outpatient clinics in Jakarta, Indonesia. Its limitations include a modest sample size, use of data from clinical records, and incomplete coverage with the 3 screening methods applied. However, the novel tests are compared with an established screening protocol (BPNS) and with risk genotypes based on *CaMKK2*. Our data link *CAMKK2* genotypes with small rather than large fiber damage, but SSW was shown to reflect pathology distinct from that identified using any component of BPNS. NC was affected by persistent HIV replication but not by *CAMKK2* genotypes. However, it may be affected by genes upstream of *CAMKK2* that have potential to influence neuronal death.

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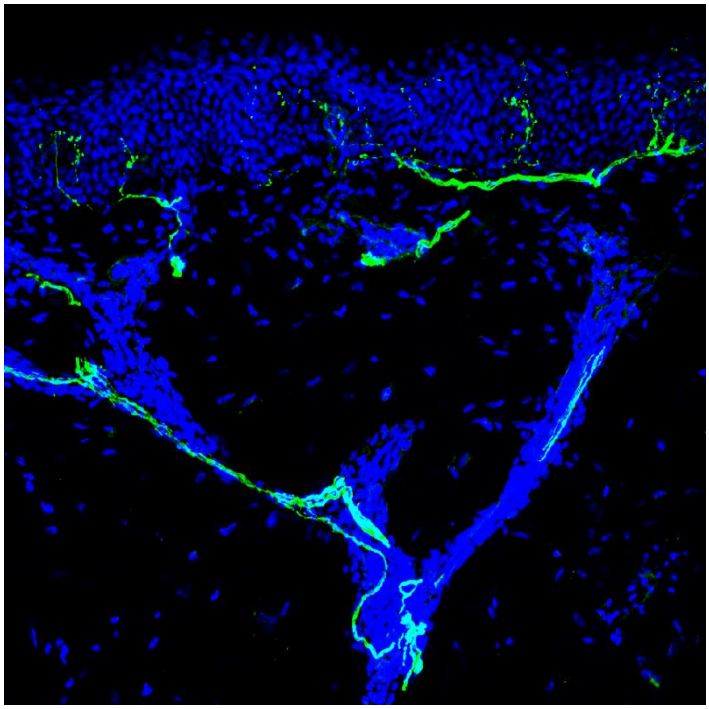
Chapter 6

Epidermal investigation of calcium/calmodulin-dependent kinase kinase 2 and purinergic receptor 7 and 4 expression in HIV-associated sensory neuropathy

Polymorphisms in the P2X-block associated with HIV-SN in Indonesians and Africans treated without stavudine. Although causative alleles have not been identified associations implicates the encoded proteins in the pathogenesis of HIV-SN. Here, I used skin biopsies to compare expression of P2X7R, P2X4R and CaMKK2 in the dermis and epidermis from Indonesian patients with and without HIV-SN, and healthy controls.



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Epidermal expression of calcium/calmodulin-dependent kinase kinase 2 and purinergic receptor 7 and 4 in HIV-associated sensory neuropathy

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ABSTRACT

Objectives: HIV-associated sensory neuropathy (HIV-SN) is a common, sometimes painful, neurological complication affecting 14-38% of HIV+ individuals treated with antiretroviral therapies excluding neurotoxic stavudine (ART). Polymorphisms in adjacent genes *P2X7R*, *P2X4R* and *CAMKK2* associate with altered risk of HIV-SN.

Design: We assessed expression of P2X7R, P2X4R and CaMKK2 proteins in skin biopsies from HIV+ Indonesians with and without HIV-SN treated for >12 months with stavudine-free ART and healthy controls (HC).

Methods: HIV-SN was assessed using the Brief Peripheral Neuropathy Screen. Biopsies from the lower leg were stained to detect protein gene product 9.5 (PGP9.5) on nerve fibres and P2X7R, P2X4R or CaMKK2, and visualised using 3-colour confocal microscopy. Intraepidermal nerve fibre density (IENFD), and expression of the three proteins was assessed in de-identified images.

Results: IENFD was lower in HIV+ donors than in HC, but similar in HIV-SN+ and HIV-SN– donors (p=0.19). IENFD correlated positively with nadir CD4 T-cell counts (r=0.69, p=0.004). P2X7R was expressed by cells in blood vessels of HIV-SN− donors, but rarely in HC or HIV-SN+ donors. P2X4R expression by cells in the epidermal basal layer appeared greatest in HIV-SN+ donors. CaMKK2+ cells were rare in HC. HIV-SN− donors had fewer CaMKK2+ cells than HIV-SN+ donors. CaMKK2+ cells were located close to dermal and epidermal nerve fibres.

Conclusions: Cells expressing P2X7R, P2X4R and CaMKK2 may interact with and damage dermal and epidermal nerve fibres, leading to a reduced IENFD and the development of HIV-SN in HIV+ Indonesians treated with ART excluding stavudine.

Word Count: 245 words

Keywords: HIV-associated sensory neuropathy, CaMKK2, P2X7R, P2X4R, Intraepidermal nerve fibre density, HIV+ Indonesians

INTRODUCTION

HIV-associated sensory neuropathy (HIV-SN) is a debilitating neurological complication of HIV infection and antiretroviral therapy (ART) [1-4]. Despite the use of ART free of drugs associated with neurotoxicity (usually stavudine), HIV-SN continues to impact up to 38% of people living with HIV [2, 3]. Symptoms of HIV-SN include numbness, "pins and needles", disordered sensation and neuropathic pain, which impact an individual's ability to work and their quality of life [5]. No interventions prevent or reverse HIV-SN progression [5], so a better understanding of the underlying mechanisms will have clinical value.

Clinical features of HIV-SN include neuronal loss and degeneration of long axons in a "die-back" manner, correlating with reduced intraepidermal nerve fibre density (IENFD) [6]. Increased infiltration of mononuclear cells and cytokine expression in dorsal root sensory ganglia (DRG) [7, 8], and increased expression of chemokine receptors by CD3+ and CD14+ cells surrounding intraepidermal nerves [9], suggest a central role for inflammation in the manifestation of HIV-SN. An inflammatory aetiology is supported by genetic associations with polymorphisms in a block of genes surrounding the *TNF* gene [10, 11], and in three neighbouring genes; *P2X7R*, *P2X4R* and *CAMKK2* (the *P2X*-block) [12-15]. Our studies associated single nucleotide polymorphisms (SNP) and haplotypes from the *P2X*-block with HIV-SN in Indonesian and South African patients, implicating the encoded proteins in the pathogenesis of HIV-SN [12, 13]

P2X7R and *P2X4R* encode purinergic P2X receptors 7 and 4 (P2X7R and P2X4R, resp.) which are activated by adenosine triphosphate (ATP) and are involved in inflammatory and neurotransmission pathways [16]. Activation of P2X7R in microglia in the DRG and satellite glial cells in spinal dorsal horn induces pro-inflammatory interleukin-1 beta (IL-1β), IL-6 and tumour necrosis factor-alpha (TNFα) via p38-mitogen activated protein kinase (p38-MAPK) [17]. Mice treated with P2X7R antagonists display reduced expression of IL-1β and IL-6 and alleviated mechanical allodynia in a model of neuropathic pain [18]. P2X4R is also implicated in the

development of neuropathic symptoms in rodent models [19]. Following spinal injury, expression of P2X4R is upregulated in the spinal cord. Intraspinal administration of P2X4R antisense oligodeoxynucleotides reduced P2X4R expression and inhibited tactile allodynia following nerve injury, and intraspinal administration of P2X4R-positive microglia triggered tactile allodynia [19]. Upregulation of P2X4R results in p38-MAPK-dependent release of brain-derived neurotrophic factor (BDNF), IL-1β, TNFα and IL-6, leading to neuropathic pain [20, 21].

CAMKK2 encodes calcium/calmodulin-dependant kinase kinase 2 (CaMKK2) which phosphorylates adenosine monophosphate (AMP)-activated protein kinase (AMPK), sirtuin 1 (SIRT1), and calcium/calmodulin-dependant kinase 1 and 4 (CaMKIV and CaMKI) [22-24]. AMPK activation mediates inflammation and apoptosis [25, 26]. SIRT1 activation regulates axonal regeneration, promotes dendrite arborisation, and protects neurons from oxidative stress [27-29]. CaMKIV activation upregulates nuclear factor κB and cyclic AMP response element-binding protein which stimulates BDNF, promoting neuronal growth and survival [23, 30, 31]. Excessive BDNF expression is implicated in neuropathic pain [20]. Activated CaMKI regulates axonal growth cone morphology and outgrowth, dendrite arborisation and synapse formation [32, 33].

Experimental evidence implicates P2X7R, P2X4R and CaMKK2 expressed by cells in DRG, spine and brain in neurological outcomes affecting the periphery. It is also plausible that these proteins may contribute directly to a reduced IENFD and the pathogenesis of HIV-SN via the degeneration of sensory nerve terminals in the skin. P2X7R is expressed in the skin by keratinocytes, Langerhans cells, dermal dendritic cells, T-cells and macrophages [34]. P2X4R expression has been detected in cultured keratinocytes, macrophages and sensory axon terminals [35-38], and CaMKK2 is expressed by monocytes/macrophages [25, 35]. Here, we assess *ex vivo* expression of P2X7R, P2X4R and CaMKK2 and their association with nerve fibres in skin biopsies from the lower leg donated by HIV+ Indonesian patients with and without HIV-SN, and healthy controls.

MATERIALS AND METHODS

Participants and Phenotyping

The study was approved by the Ethics Committee of the Faculty of Medicine, Universitas Indonesia (579/UN2.F1/ETIK/2014) and validated by Curtin University (HR210-2015). Written and informed consent was obtained from all participants. HIV+ adults who had used ART for at least 12 months (median 6.4 years; range 1.2-11.7 years), but who had never been exposed to stavudine, were screened for neuropathy at POKDISUS HIV Care Clinic, Cipto Mangunkusumo Hospital, Jakarta, Indonesia in 2016 [2]. Individuals with a history of other conditions potentially associated with a neuropathy, or any condition preventing provision of informed consent, were excluded. Neuropathy was assessed using the AIDS Clinical Trials Group Brief Peripheral Neuropathy Screen (ACTG-BPNS), and defined as present if the individual had one or more of the lower limb neuropathic symptoms (pain, aching or burning, "pins and needles" or numbness), together with absent ankle reflexes or reduced vibration sense at the great toe (vibration of a 128-Hz tuning fork felt for 10 seconds or less). We did not diagnose neuropathy in patients with only asymptomatic neuropathic signs, as the presence of both symptoms and signs on the BPNS tool better associates with impaired peripheral nerve function and pathology [39]. Biopsies were collected from six individuals with HIV-SN (HIV-SN+) and six individuals without HIV-SN (HIV-SN–). An additional five control biopsies were provided from healthy, age-matched adults of South East Asian ancestry (HC) who declared no risk factors for HIV.

Biopsy collection

Biopsies were collected as published previously [9]. Briefly, 2% lidocaine with epinephrine anaesthesia was injected subcutaneously under sterile conditions approximately 10cm above the lateral malleolus. The skin biopsy was collected using a 3mm circular skin punch, fixed in 4% paraformaldehyde-lysine-periodate overnight at 4°C, and incubated with cryoprotectant (20% glycerol and 80% 0.1M Sorenson's phosphate buffer) overnight at 4°C prior to storage at -20°C. Biopsies were sectioned at 50µm using a freeze cryostat sliding microtome (Leica Biosystems,

Nussloch, Germany) and stored in antifreeze (33% glycerol, 33% ethylene glycol, 10% 2x phosphate buffer, 24% dH_2O) at -20°C.

Immunofluorescent protocol

Floating sections were incubated with Image-iT FX Signal Enhancer (Invitrogen, Carlsbad, CA, USA) for 30 minutes (min) at room temperature, incubated over consecutive nights at 4°C with primary, secondary and detection antibodies diluted in Tris buffered saline (TBS) with 5% normal donkey serum (NDS), and washed the following morning six times for 1 hour each at room temperature using TBS with 0.01%v/v triton-X (TBS-wash). Sections were treated with goat anti-CaMKK2 (sc-9629; 2 µg/ml; Santa Cruz Biotechnology, Dallas, TX, USA), P2X4R (ab134559; 5 µg/ml; Abcam, Cambridge, UK) or P2X7R (ab105047; 5 µg/ml; Abcam), followed by biotinylated donkey anti-goat IgG (ab6884; 20 µg/ml; Abcam) and detected with streptavidin conjugated to AlexaFluor™ 647 (S-32357; 20 µg/ml; Invitrogen). Protein gene product 9.5 (PGP9.5) was targeted using rabbit anti-PGP9.5 (ab15503; 2 µg/ml; Abcam) and detected with mouse anti-rabbit IgG conjugated with Dylight® 594 (ab96893; 5 µg/ml; Abcam). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; 1:10,000; Invitrogen) for 10 min at room temperature to visualise tissue morphology. Sections were washed twice with TBS-wash, and mounted on glass slides (Proscitech, Queensland, Australia) with a coverglass (#1.5; Proscitech) using Immumount (Thermo Scientific, Waltham, MA, USA). Section treated without primary antibodies were included as negative controls (Supplementary Figure 1).

Microscopy

Samples were imaged using an inverted Nikon A1+ confocal microscope (Nikon Instruments, New York, NY, USA) and NIS-Elements software (Nikon Instruments). Images were acquired using a digital scan resolution of 0.64µM/pixel, pinhole of 1.2 airy units and pixel dwell 6.2 with 1024 resolution using a 20x Plan Apo dry objective (N.A. 0.75; Olympus Corporation, Tokyo, Japan). Sequential scanning was completed using three lasers; 405nm (450/50 filter), 561nm

(595/50 filter) and 640 nm (700/75 filter) to view nuclei, PGP9.5+ nerve fibres and P2X4R+, P2X7R+ and CaMKK2+ cells respectively. Multiple images spanning 0.5mm were acquired across the full width of the epidermis in a z-series at 1.1 µm intervals (as per Nyquist settings) through the full thickness of the section. Z-series for each section were combined into a single maximum intensity projection image with NIS-Elements Viewer (Nikon Instruments) and equivalent thresholds were applied across all images to assess PGP9.5+ nerve fibres (red) and P2X7R, P2X4R and CaMKK2 positive cells (green).

P2X7R, P2X4R and CaMKK2 expression and intraepidermal nerve fibre density

The expression patterns of P2X7R, P2X4R or CaMKK2 and association with PGP9.5+ nerve fibres was investigated from a subset of HC (n=3), HIV-SN– (n=3) and HIV-SN+ (n=3) donors selected randomly for each protein. Images from three sections per donor were assessed and expression patterns described.

Three images from all HC (n=5), HIV-SN– (n=6) and HIV-SN+ (n=6) donors were analysed to determine the IENFD. Images were available for an additional two HC, three HIV-SN– and two HIV-SN+ donors assessed for IENFD in 2017 using the same study criteria and protocols, and were included here to better identify differences between donor groups [2, 9]. All images were coded and PGP9.5+ nerve fibres were quantitated by three raters (JG, PK and PP) blinded to donor diagnoses. Quantitation was completed using a standard IENFD protocol [40] in which single fibres crossing the dermo-epidermal junction are counted and secondary branches are excluded. The average counts derived from each sample were doubled to generate IENFD per square millimetre. Fisher's exact tests, Mann-Whitney tests, and Spearman's correlations were used as appropriate to compare IENFD with donor characteristics using GraphPad Prism version 8.2.1 for Windows (Graphpad Software, La Jolla, CA, USA). An intraclass correlation coefficient was calculated (two-way random, single measures and absolute agreement) using the "irr" package [41] in the R statistical environment [42].

RESULTS

Intraepidermal nerve fibre densities correlated with nadir CD4 T-cells/µl but did not differ between donors with and without HIV-SN

Confocal images of PGP9.5+ intraepidermal nerve fibres were collected (Supplementary Figure 2) and IENFD determined for all donors (Table 1). The intraclass correlation coefficient was 0.86 (95% confidence interval = 0.79-0.92), suggesting strong agreement between the three raters (data not shown). IENFD was higher for HC than those with or without HIV-SN (p = 0.02 and 0.07, resp.), but similar in HIV-SN+ and HIV-SN– donors (p=0.19; Table 1). There were no differences in age, height, time on ART and current CD4 T-cells between HIV-SN– and HIV-SN+ donors (Supplementary Table 1). However, nadir CD4 T-cell counts were lower in HIV+ individuals with HIV-SN than those without (p=0.05; Table 1). To explore this difference, we assessed whether IENFD correlated with demographic and clinical features of donors and determined that IENFD correlated positively with nadir CD4 T-cell counts (r=0.67, p=0.004; Supplementary Figure 3). Age did not correlate with IENFD (r=-0.36, p=0.08).

P2X7R+ cells were observed in dermal blood vessels of HIV-SN– donors, but rarely in HC or HIV-SN+ donors

P2X7R was variably expressed across donor groups, but was very rare in sections from HC (Figure 1a). Expression was also rare in sections from HIV-SN+ donors, but a few P2X7R+ cells were observed in dermal blood vessels (yellow arrows) and some within the epidermis close to epidermal nerves (yellow box; Figure 1c). Sections from two out of three of the HIV-SN– donors exhibited an abundance of P2X7R+ cells in dermal blood vessels (Figure 1b). Sections from the remaining HIV-SN– donor had moderately higher levels of P2X7R expression in blood vessels than HC and HIV-SN+ donors (Supplementary Table 1, donor #18). P2X7R+ cells were observed close to epidermal nerve fibres in sections from all three HIV-SN– donors (Figure 1b). This suggests up-regulation by HIV disease – potentially as a mechanism that suppresses HIV-SN.

P2X4R expression may be increased in the epidermis of HIV-SN+ donors

P2X4R+ cells were present in dermal blood vessels and the basal layer of the epidermis in all sections from all three HC, HIV-SN– and HIV-SN+ donors assessed for P2X4R expression (Figure 2). P2X4R+ cells were often closely located to epidermal nerves and subepidermal nerve plexi (yellow arrows; Figure 2a-c). While expression of P2X4R occurred in all donors, its expression in the basal layer of the epidermis was more abundant in sections from all three HIV-SN+ donors compared to HC and HIV-SN- donors (Figure 2c vs Figure 2a and b). This is consistent with a role for P2X4R in HIV-SN.

Donors with HIV-SN had more CaMKK2+ cells than HIV-SN– donors, and CaMKK2+ cells were close to dermal and epidermal nerves

CaMKK2+ cells were only observed in sections from two of the three the HC donors, and even then expression was limited to a few cells (Figure 3a). CaMKK2+ cells were more evident in sections from all three HIV-SN– and HIV-SN+ donors. However, sections from HIV-SN– donors had slightly fewer CaMKK2+ cells (an average of 3, 5 and 8 positive cells per donor; Figure 3b) than HIV-SN+ donors (an average of 8, 14 and 19 positive cells per donor; Figure 3c). CaMKK2+ cells were typically located close to dermal and epidermal nerves (yellow arrows) or co-located with dermal nerves (yellow box; Figure 3c), so CaMKK2+ cells may interact with peripheral nerves in HIV+ patients.

DISCUSSION

We assessed IENFD of PGP9.5+ fibres and investigated *ex vivo* expression of P2X7R, P2X4R and CaMKK2 in skin biopsies from healthy controls, HIV-SN– and HIV-SN+ Indonesians receiving stavudine-free ART for >12 months. The median (range) IENFD for HC was 12.7 (7.4-17.3/mm^2), which is comparable to earlier studies [43, 44]. IENFD was lower in HIV+ donors compared to HC, but did not differ between donors with and without HIV-SN (p=0.19; Table 1). We are not

the first to show this. A longitudinal investigation of 150 HIV+ Thai individuals not detect a difference in IENFD between those with and without HIV-SN [45]. Lower IENFD was recently associated with the presence of neuropathic pain at the biopsy site [46]. The prevalence of neuropathic pain in Indonesian HIV-SN+ patients is low (5/28; [47]). Only one biopsy was available from an HIV-SN+ donor with neuropathic pain (IENFD = 4.4/mm^2; not shown), so we couldn't assess IENFD in relation to pain.

Age is negatively correlated with IENFD in healthy individuals [44] but has not been linked in HIV patients [6, 9, 45, 48]. Here, HIV+ participants are relatively young (aged 23 to 47) and age did not correlate with IENFD (r=-0.36, p=0.08). Instead, we identified a positive correlation between nadir CD4 T-cell counts and IENFD in the HIV+ groups (r=0.67, p=0.004; Supplementary Figure 3). We previously associated lower CD4 T-cell counts at the time of assessment with greater risk of HIV-SN in Indonesians [2], and with lower nadir CD4 T-cells in HIV+ Africans [13]. A lower CD4 T-cell count may reflect a greater severity of HIV disease, supporting a direct role for HIV itself in the degeneration of epidermal nerve fibres and highlighting the need for early initiation of ART.

CaMKK2+ cells were identified in all HIV+ sections. CaMKK2+ cells were usually located close to or co-located with damaged nerve fibres and were more common in donors with HIV-SN (Figure 3). CaMKK2 activates AMPK, a master regulator of cellular energy homeostasis. AMPK activation can replenish ATP supplies required for energy intensive axonal growth by recruiting mitochondria to the site of repair [49]. Therefore, the close association between CaMKK2+ cells and nerve fibres may reflect CaMKK2-mediated neuronal growth and repair pathways. CaMKK2 is also expressed by macrophages and mediates inflammatory pathways [25, 50]. CaMKK2 activates CaMKIV which in turn activates the p38-MAPK cascade and activation factor 1 (AP-1), inducing pro-inflammatory cytokines including TNFα, IL-1β and IL-6 [32]. It is plausible that

CaMKK2+ cells may contribute directly to a reduced IENFD and HIV-SN via neuronal or inflammatory pathways.

P2X4R expression was observed in the basal layer of the epidermis, in blood vessels and closely located to epidermal nerves and subepidermal nerve plexi in all donors assessed (Figure 2). However, P2X4R appeared upregulated in the basal layer of the epidermis in HIV-SN+ donors. Most cells in the basal layer proliferate and differentiate into keratinocytes which can express sensory receptors and produce neuroactive molecules that can illicit nociceptive responses in epidermal axon terminals of sensory neurons in response to noxious stimuli [51]. Rodent studies show that mechanical, cold, and heat stimulation of keratinocytes produces ATP which activates P2X4R receptors on sensory neurons and results in behaviours associated with temperature stress and pain [37, 38]. As extracellular ATP can act on P2X4R in an "autocrine" fashion [52], the increased expression of P2X4R in the epidermis of HIV-SN+ donors could plausibly exacerbate this nociceptive pathway and contribute to neuropathic symptoms.

P2X4R is also a moderator of inflammation [20]. Sciatic nerve injury in a rodent model of neuropathy upregulates P2X4R expression in spinal microglia leading to increased production of IL-1β, TNFα and IL-6 [20, 21]. Activation of keratinocyte P2X4R is associated with production of IL-6 [36]. Like P2X4R, P2X7R is implicated in inflammation associated with neuropathic pain [18]. Interestingly, P2X7R was abundantly expressed in the blood vessels of individuals without HIV-SN, but less in HC or HIV-SN+ individuals (Figure 1). Expression of P2X7R may be dependent on disease phenotype, disease stage and tissue or cell type [53, 54]. For example, P2X7R expression was upregulated on peripheral blood monocytes and lymphocytes in patients with neuropathic pain but not in patients with chronic nociceptive low back pain when compared to healthy controls [55]. Furthermore P2X4R and P2X7R expression may be compensatory as P2X4R was upregulated in CD4+ T-cells from P2X7R knockout mice in a model of heart transplantation [56].

Our study has limitations. Firstly, we acknowledge the modest number of donors. However, a well-established protocol for determining IENFD [40] and the assessment of IENFD by multiple raters blinded to donor diagnoses ensured reliable quantification. This was supported by an intraclass correlation coefficient of 0.86 (95% confidence interval = 0.79-0.92) indicating strong agreement between raters. Secondly, we utilised a simple clinical tool to diagnose HIV-SN. We cannot rule out that HIV-SN– donors may have had sub-clinical peripheral nerve pathology or physiological malfunctions not detected by this tool. We assessed if IENFD correlated with large or small fibre neuropathy diagnosed in a subset of participants using established nerve conduction tests and stimulated skin wrinkling tests, respectively [15]. However, as with HIV-SN diagnosed by BPNS, IENFD did not differ between donors with and without large or small fibre neuropathies (0.28 and p=0.62, resp.; Online Supplementary Table 2). Finally, no data pertaining to the duration of neuropathy were available due to the cross-sectional design. This may explain individual variation of P2X4R, P2X7R or CaMKK2 expression within the donor groups.

Overall, we have demonstrated that IENFD is reduced in HIV+ participants, and IENFD is positively correlated with nadir CD4 T-cells which may reflect an individual's severity of HIV disease prior to commencing ART. We identified that P2X4R is upregulated in epidermal basal layer cells in donors with HIV-SN whereas P2X7R+ cells were more abundant in the blood vessels of HIV+ donors without HIV-SN. Finally, we demonstrated that tissue from donors without HIV-SN contained fewer CaMKK2+ cells compared to donors with HIV-SN, and most CaMKK2+ cells were located near or co-located with PGP9.5+ nerves. The expression patterns and location of P2X7R, P2X4R and CaMKK2+ cells suggests a role for these proteins in the pathogenesis of HIV-SN in the periphery.

Table 1. Summary of donor characteristics

BPNS Diagnosis	Male	Age (years)	Height (cm)	Time on ART (months)	Nadir CD4 (cells/µl)	Current CD4 (cells/µl)	IENFD (per mm^2)
HC	2/7	33 (23-41)	-	-	-	-	12.7 (7.4-17.3)
HIV-SN–	4/9	36 (25-44)	165 (150-179)	58.6 (14.8-141)	225 (6-330)	435 (84-693)	5.2 (3.5-18.4)
HIV-SN+	4/8	36 (29-47)	167 (155-175)	84.4 (24.4-131)	47 (17-166)	485 (284-729)	3.8 (1.3-15.4)
	P=0.99 [a]	P=0.83 [b]	P=0.63 [b]	P=0.33 [b]	**P=0.05** [b,c]	P=0.67 [b]	P=0.19 [b]

Results are presented as median (range)

[a] Fisher's exact test (HIV-SN+ *versus* HIV-SN-);

[b] Mann-Whitney test (HIV-SN+ *versus* HIV-SN-)

[c] P≤0.05 are in bold

BPNS, brief peripheral neuropathy screening tool; ART, antiretroviral therapy; IENFD, intraepidermal nerve fibre density

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Author Contributions

JG conducted lab work, statistical analyses and wrote the manuscript; FO recruited participants, collected medical data, conducted neuropathy assessments and collected biopsies; SL assisted with lab work and provided advice for analyses; CJ assisted with sample preparation and provide microscopy expertise; PK provided statistical advice and assisted with analyses; JP provided pathology expertise; JM assisted with the collection and processing of biopsies, assisted with protocol design and provided the additional images for analyses; PP assisted with analyses and coordinated the project.

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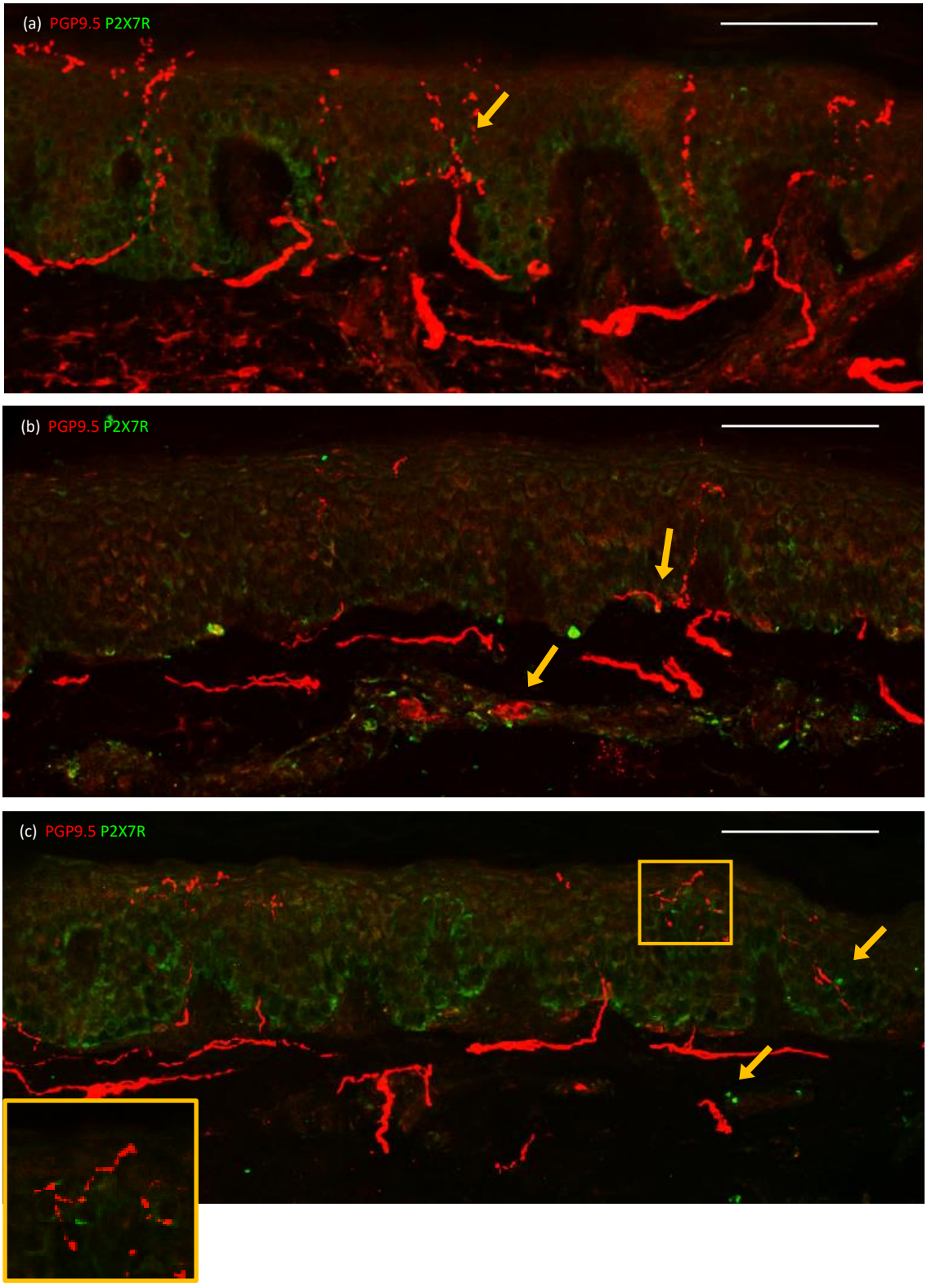


Figure 1. Representative confocal images showing intraepidermal expression of PGP9.5 (red) and P2X7R (green) in HC (a), HIV-SN– (b), HIV-SN+ (c). P2X7R+ cells (yellow arrows) are rarely seen in sections from HC donors (a). P2X7R+ cells (yellow arrows) are abundant in dermal blood vessels and sometimes closely located to epidermal nerves of sections from HIV-SN– donors (b). P2X7R+ cells are occasionally seen in dermal blood vessels and closely located to epidermal nerves (yellow box) in HIV-SN+ donors (c). scale bar = 100 µm

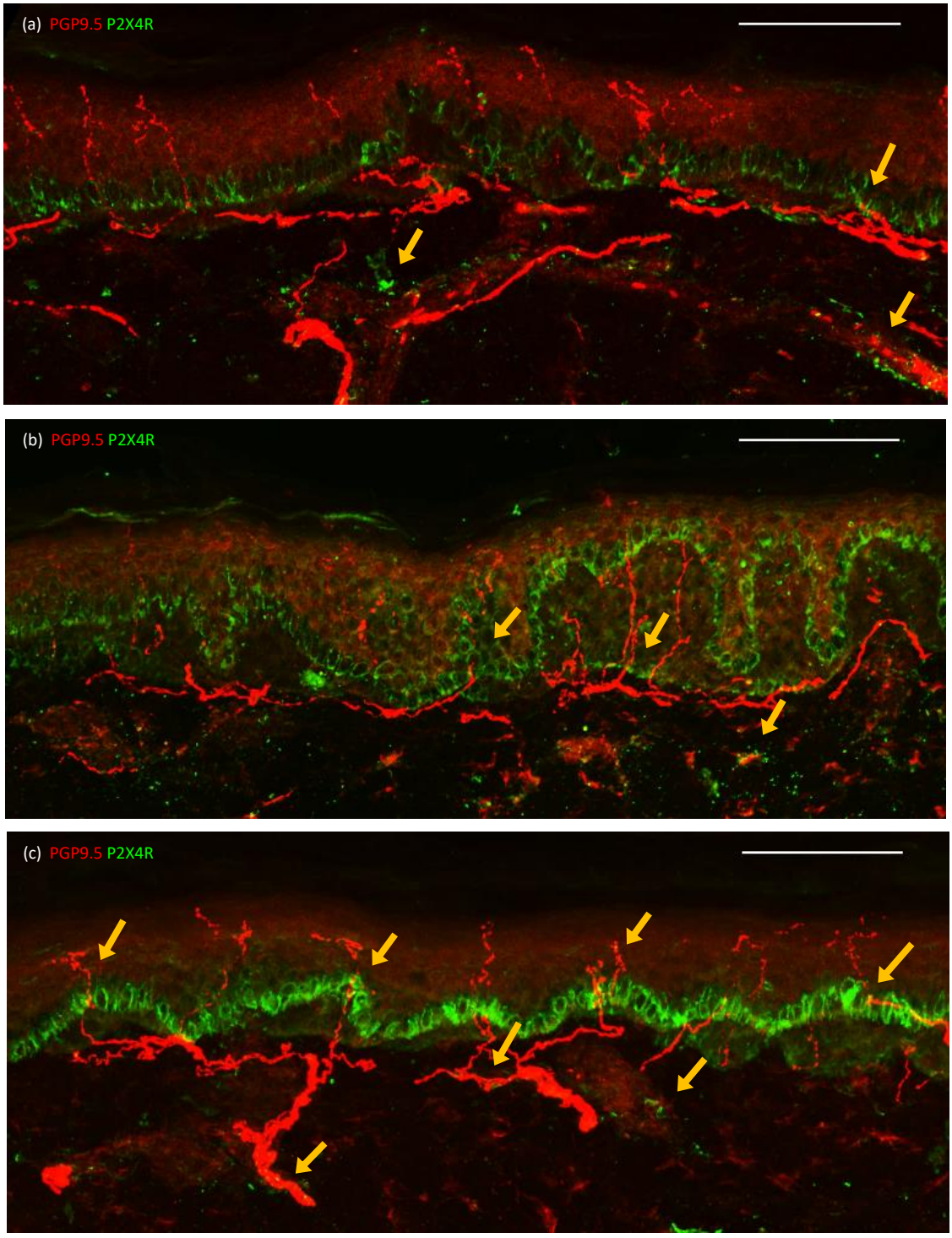


Figure 2. Representative confocal images demonstrating intraepidermal expression of PGP9.5 (red) and P2X4R (green) in HC (a), HIV-SN– (b), HIV-SN+ (c). P2X4R expression is observed in the basal layer of the epidermis and dermal blood vessels of all donors but was increased in sections from donors with HIV-SN (c). P2X4R+ cells were observed in dermal blood vessels in all donors and located near dermal and epidermal nerves (yellow arrows). scale bar = 100 µm

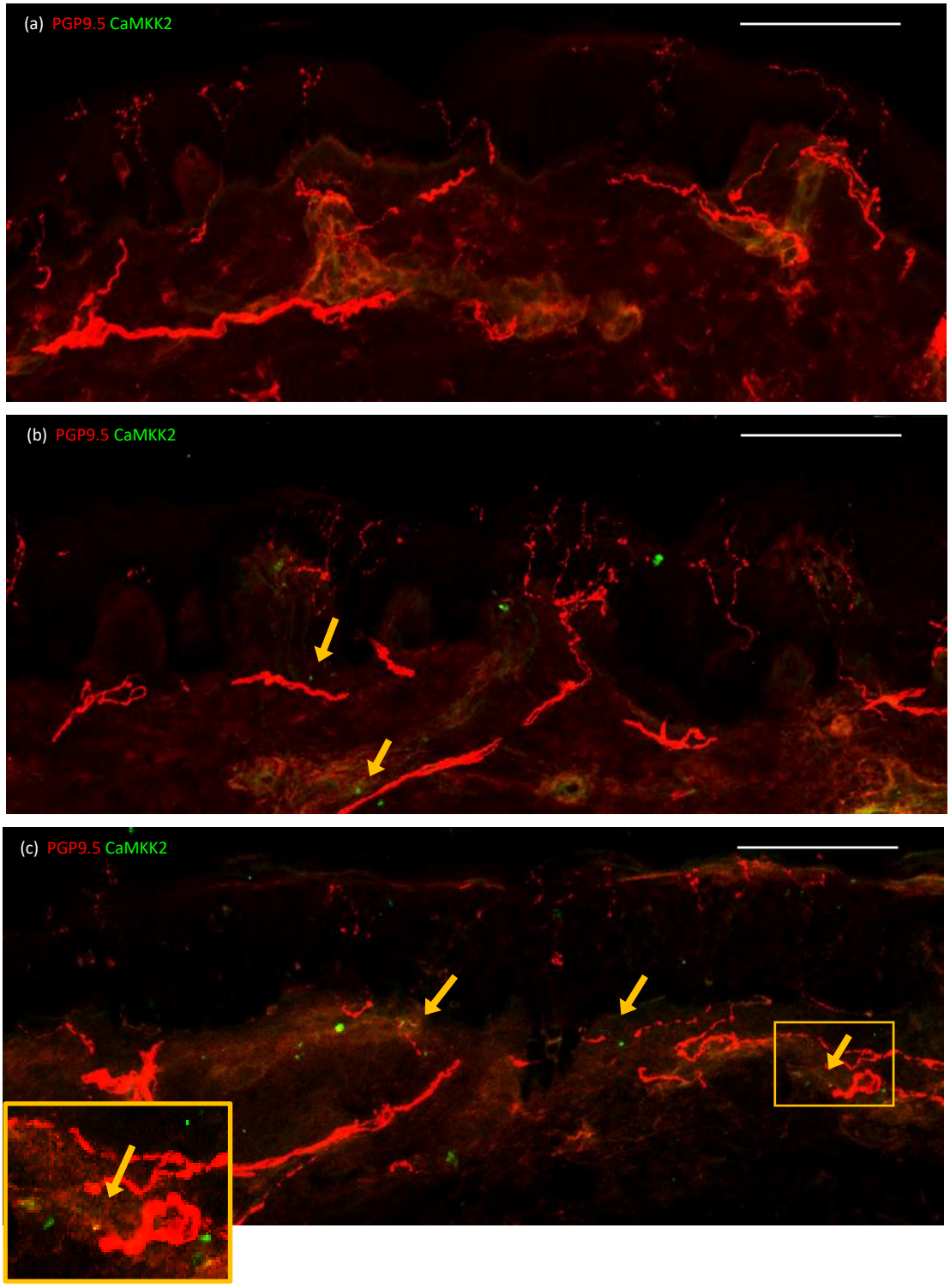
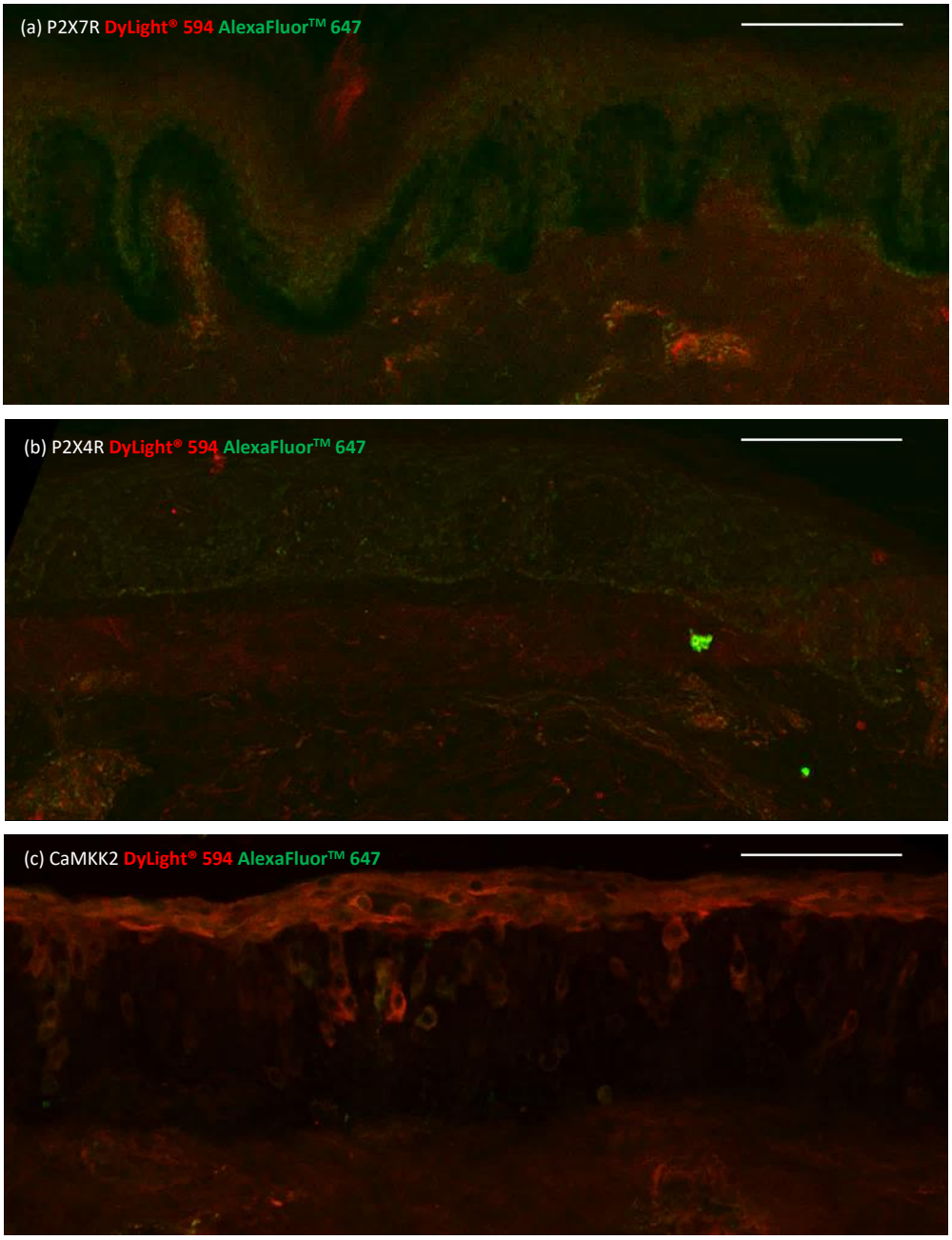
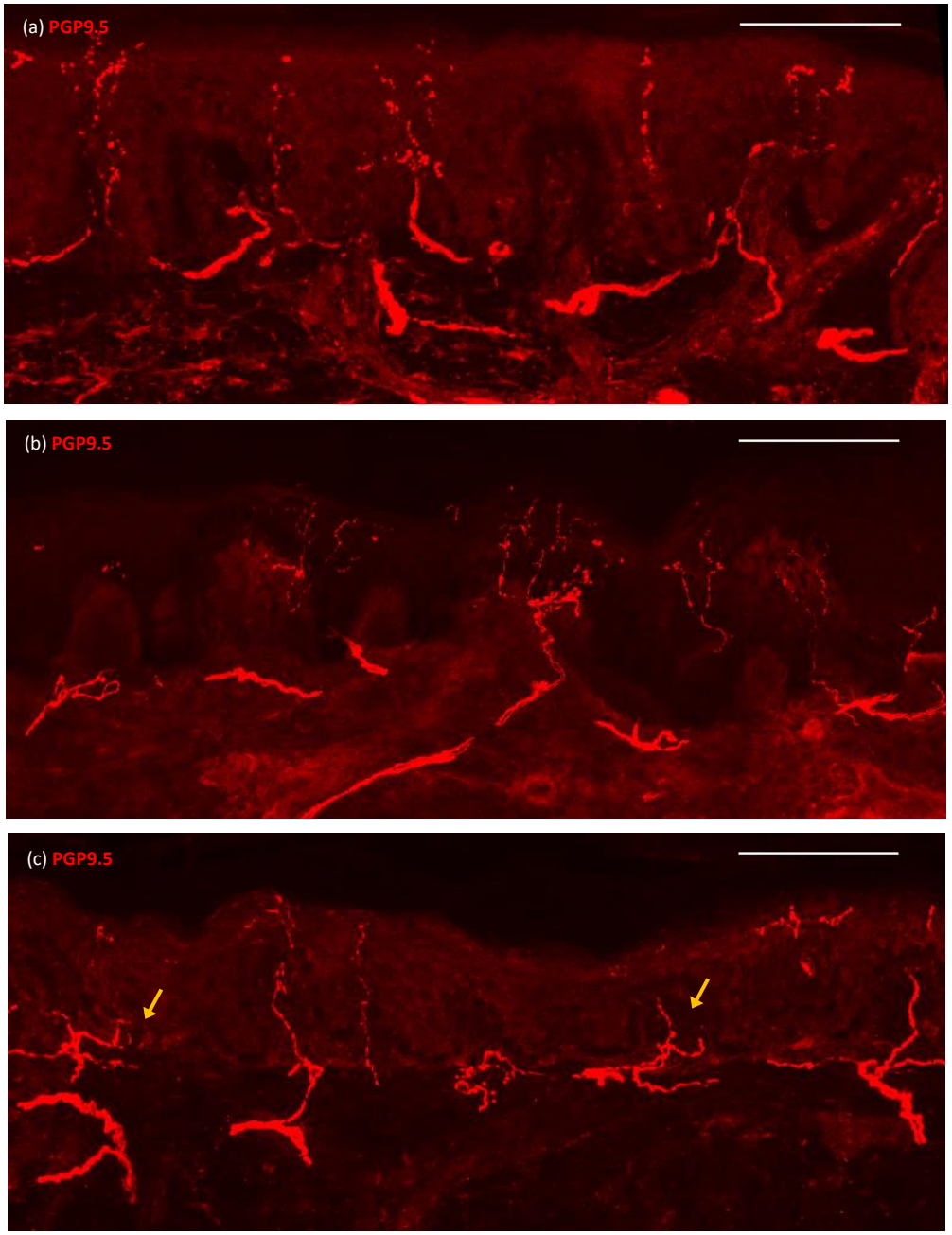


Figure 3. Representative confocal images showing intraepidermal expression of PGP9.5 (red) and CaMKK2 (green) in HC (a), HIV-SN– (b), HIV-SN+ (c). CaMKK2+ cells are very rarely seen in sections from HC donors (a) but are observed in sections from HIV-SN– and HIV-SN+ donors (b-c). CaMKK2+ cells are usually located close to nerves (yellow arrows; b-c) or co-located with nerves - appearing yellow (yellow box; c). scale bar = 100 µm

Online Supplements



Supplementary Figure 1. Representative confocal images of negative control sections for P2X7R (a), P2X4R (b) and CaMKK2 (c) treated only with secondary antibodies DyLight® 594 and AlexaFluor™ 647.
Background staining of Dylight® 594 (red) and AlexaFluor™ 647 (green) occurred in the dermis and blood vessels of all negative controls (a-c). Scale bar = 100µm



Supplementary Figure 2. Representative confocal images of PGP9.5+ intraepidermal nerve fibres from HC (a), HIV-SN- (b) and HIV-SN+ (c) donors. Numerous PGP9.5+ fibres branch from dermal fibres innervating the epidermis in HC donors (a) with a median (range) intraepidermal nerve fibre density of 12.7 (7.4-17.3). The number of PGP9.5+ fibres were similar in HIV-SN– (5.2 [3.5-18.4]) and HIV-SN+ donors (3.8 [1.3-15.4]). A reduction in epidermal fibres length was common in HIV-SN+ donors (yellow arrows; c). Scale bar = 100µm

Supplementary Table 1. Characteristics of IENFD donors

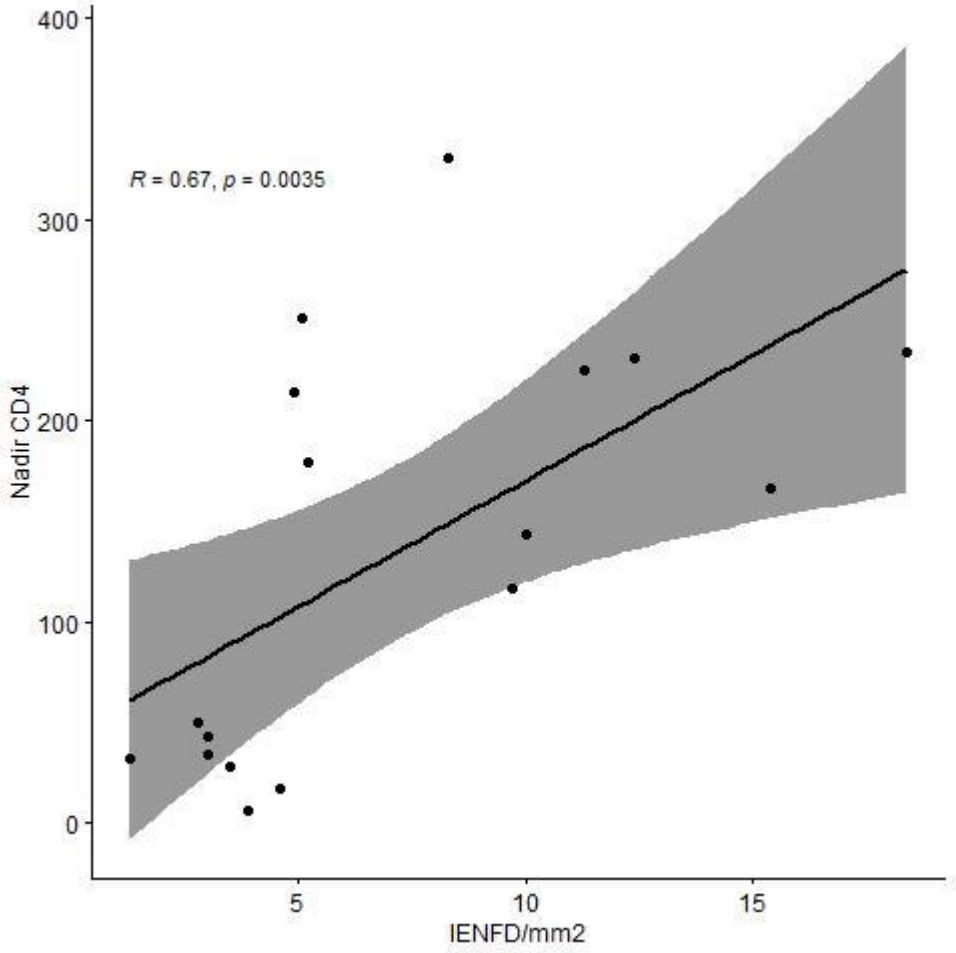
#	HIV-SN[a]	Small fibre [b]	Large fibre [c]	Sex	Age (years)	Height (cm)	Nadir CD4 (cell/µL)	Last CD4 (cell/µL)	Time on ART (months)	IENFD (per mm^2)
1	HC	-	-	Female	26	-	-	-	-	16.3
2	HC	-	-	Female	41	-	-	-	-	16.0
3	HC	-	-	Female	30	-	-	-	-	17.3
4	HC	-	-	Female	33	-	-	-	-	12.7
5	HC	-	-	Female	33	-	-	-	-	9.7
6	HC	-	-	Male	37	-	-	-	-	10.2
7	HC	-	-	Male	23	-	-	-	-	7.4
8	Neg	NA	Neg	Female	40	150.5	251	448	76.8	5.1
9	Neg	Neg	Neg	Female	32	165	225	385	42.7	11.3
10	Neg	Neg	Neg	Female	36	153	234	321	84.4	18.4
11	Neg	Pos	Neg	Female	41	150	330	435	24.1	8.3
12	Neg	Pos	Neg	Male	31	174	214	386	58.6	4.9
13	Neg	Neg	Neg	Male	44	171	6	84	27.6	3.9
14	Neg	Neg	Neg	Male	38	179	179	626	103.7	5.2
15	Neg	Pos	NA	Male	35	165	28	693	140.7	3.5
16	Neg	Pos	Neg	Male	25	165	231	653	14.8	12.4
17	Pos	Neg	Neg	Female	37	155	43	526	92.2	3.0
18	Pos	Neg	Neg	Female	29	163	166	598	87.8	15.4
19	Pos	Neg	Pos	Female	32	167	117	729	47.4	9.7
20	Pos	Pos	Pos	Female	31	158	50	406	131.1	2.8
21	Pos	Pos	Pos	Male	47	167	17	284	80.9	4.6
22	Pos	Neg	Neg	Male	34	175	34	714	58.6	3.0
23	Pos	Neg	Neg	Male	41	171	143	444	110	10.0
24	Pos	Neg	NA	Male	45	167	32	300	24.4	1.3

[a] HIV-SN diagnosis using BPNS

[b] Small fibre neuropathy diagnosis using stimulated skin wrinkling tests [15]

[c] Large fibre neuropathy diagnosis using nerve conduction tests [15]

HC, healthy control; NA, not available; Pos, positive test results; Neg, negative test result; ART, antiretroviral therapy; IENFD, intraepidermal nerve fibre density



Supplementary Figure 3. Nadir CD4 T-cell counts positively correlated with IENFD

Spearman's correlation coefficient

Supplementary Table 2. IENFD was generally lower in BPNS+ than BPNS- participants

Diagnosis of Donors	IENFD (per mm^2)		P value [b]
	Positive Donors [a]	Negative Donors [a]	
HIV-SN	3.8 (1.3-15.4) n=8	5.2 (3.5-18.4) n=9	0.19
Large fibre neuropathy	4.6 (2.8-9.7) n=3	8.0 (3.0-18.4) n=13	0.29
Small fibre neuropathy	4.8 (2.8-12.4) n=6	7.5 (1.3-18.4) n=10	0.62

[a] Median (Range)
[b] Mann-Whitney test

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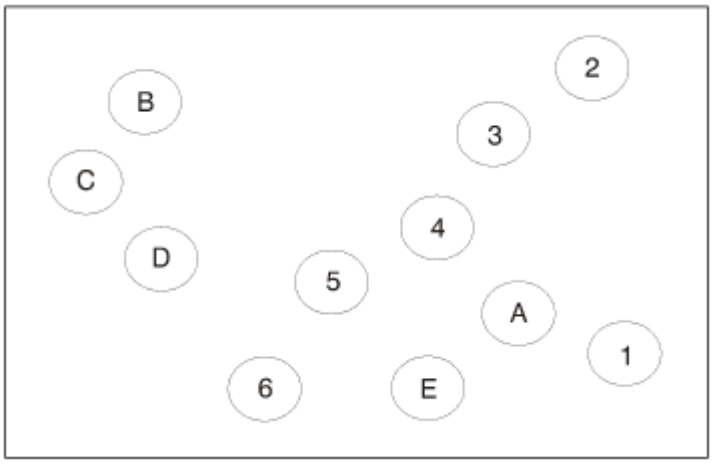
Chapter 7

Neurocognitive outcomes in Indonesians living with HIV are influenced by polymorphisms in the gene encoding purinergic P2X receptor 7

P2X-block genotypes associated with HIV-SN. HIV-associated neurological disorder (HAND) is a spectrum of neurological disorders resulting from HIV infection. HAND has a clinical pathology including inflammation and neuronal degeneration in the brain. It is plausible that inflammatory and neuronal repair mechanisms contributing to HIV-SN in the periphery may also contribute to the pathogenesis of HAND. Here I assessed associations between P2X7R SNP and neurocognitive outcomes in HIV+ Indonesians across five neurocognitive domains, over the first 12 months of stavudine-free ART.



Trail Making Test B – Assessing Executive Function

Data from this chapter have been published:

Gaff J, Estiasari R, Diafiri D, Halstrom S, Kamerman P, and Price P**. Neurocognitive outcomes in Indonesians living with HIV are influenced by polymorphisms in the gene encoding purinergic P2X receptor 7.** Brain, Behavior, & Immunity - Health. 2021;13:100220. doi: 10.1016/j.bbih.2021.100220

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Full Length Article

Neurocognitive outcomes in indonesians living with HIV are influenced by polymorphisms in the gene encoding purinergic P2X receptor 7

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ABSTRACT

The advent of effective antiretroviral therapy (ART) has decreased the prevalence and severity of HIV-associated neurocognitive disorders (HAND), but milder forms of HAND remain despite optimal treatment. Neuronal injury and loss due to inflammation may mediate HAND. *P2X7R* encodes purinergic P2X receptor 7 which influences neuroinflammatory pathways and carries polymorphisms associated with sensory neuropathy in HIV patients. We assessed associations between *P2X7R* polymorphisms and neurocognitive outcomes in Indonesian patients (n = 59) as they commenced ART and after 3, 6 and 12 months. Z-scores were calculated over 5 domains using local controls and evaluated as continuous variables. Optimal linear regression models identified polymorphisms influencing attention, memory, executive function, motor speed and total cognitive function at each time point. rs504677 was associated with lower executive and motor speed Z-scores at 0, 3, 6, and 12 months, and with memory at 0 and 12 months. Memory was positively influenced by carriage of the rs208296 minor allele at 0, 3 and 6 months and by carriage of the rs208307 minor allele at 0 and 12 months. Higher attention Z-scores associated with carriage of minor alleles of rs1653598 after 0 and 12 months. These also positively influenced executive function and motor speed after 0–6 months. This study identifies polymorphisms in *P2X7R* which influence domain-specific neurocognitive outcomes in HIV+ Indonesians prior to and shortly after commencing ART. This implicates purinergic P2X receptor 7 in the pathogenesis of HAND.

1. Introduction

Effective antiretroviral therapy (ART) has decreased the prevalence and severity of HIV-associated neurocognitive disorders (HAND), but milder forms of HAND remain a serious complication of HIV infection (Estiasari et al., 2015; Heaton et al., 2010a; Cysique et al., 2014). Neurocognitive impairment affects up to 50% of ART naive Indonesian patients with <200 CD4 T-cells/μl, and improvements in neurocognitive function after 6 months of ART are influenced by age, education and CD4 T-cells/μl (Estiasari et al., 2015, 2020). In vivo and in vitro studies of the neuropathology of HAND identify neuroinflammation as a crucial mediator of neurocognitive impairment [reviewed in (Ru and Tang, 2017)]. HIV-infected monocytes and CD4 T-cells cross the blood-brain barrier, resulting in infection and activation of microglia and astrocytes. These cells release proinflammatory cytokines and chemokines including tumor necrosis factor-α (TNFα), interleukin-6 (IL-6), interleukin-1β (IL-1β), chemokine (C–C motif) ligand 2 (CCL2), and viral proteins including transactivator of transcription (Tat) and glycoprotein 120 (gp120), resulting in release of adenosine triphosphate (ATP), intracellular influx of calcium ions (Ca^{2+}), and oxidative stress. This leads to further inflammation and neuronal and synaptic dysfunction characteristic of neurocognitive impairment and HAND (Ru and Tang, 2017).

Purinergic P2X receptor 7 (P2X7R) is an ATP-gated non-specific cation channel involved in neuroinflammatory pathways [reviewed in (Alves et al., 2020)]. P2X7R is highly expressed in the brain and is activated by high levels of ATP released from damaged cells, triggering

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an influx of Ca^{2+} and efflux of potassium ions (K^+) (Alves et al., 2020). P2X7R-dependent intracellular depletion of K^+ drives the assembly of the nucleotide-binding domain (NOD)-like receptor protein 3 (NLRP3) inflammasome, promoting the accumulation of caspase-1 which cleaves pro-IL-1β and releases mature IL-1β (Giuliani et al., 2017). P2X7R activation induces NADPH oxidase-dependent production of IL-6 from astrocytes (Munoz et al., 2020) and the release of TACE (TNFα converting enzyme) which subsequently increases release of TNFα from microglia (Barberà-Cremades et al., 2017). Moreover, in a rodent model of cognitive dysfunction induced by gp120, P2X7R expression was significantly higher in the hippocampus compared to control groups (Liu et al., 2017). In rat primary cultured microglia, application of gp120 stimulated P2X7R expression, increased concentrations of TNFα and IL-1β, and resulted in microglial apoptosis (Chen et al., 2017). In human astrocyte and neuron cultures, treatment with Tat resulted in calcium-dependent upregulation of P2X7R, release of CCL2 from astrocytes, and direct and indirect neuronal death (Tewari et al., 2015).

The gene encoding P2X7R (*P2X7R*) is highly polymorphic and located in a region of high linkage disequilibrium (LD) on chromosome 12. We have associated single nucleotide polymorphisms (SNP) and haplotypes within *P2X7R* with HIV-associated sensory neuropathy (a common neurological complication of HIV infection affecting peripheral nerves) in Indonesians and Africans (Gaff et al., 2019a, 2020; Goullee et al., 2016; Safri et al., 2020). Furthermore, SNP in *P2X7R* have been associated with neuroinflammatory and neuropsychological conditions including bipolar disorder, multiple sclerosis, and Alzheimer's disease (Sanz et al., 2014; Oyanguren-Desez et al., 2011; McQuillin et al., 2009). In the present study, we assessed associations between SNP in *P2X7R* and neurocognitive outcomes across five neurocognitive domains in HIV+ Indonesians as they commenced ART and after 3, 6 and 12 months.

2. Materials and methods

2.1. Participants

The JakCCANDO study conducted in Cipto Mangunkusumo National General Hospital, Jakarta, Indonesia (Wulandari et al., 2017) recruited 82 adults with horizontally-acquired HIV infection (Estiasari et al., 2015, 2020), with <200 CD4 T-cells/μl, a Karnofsky performance score 70–100, living in Jakarta, and providing written and informed consent to participate in the study. Participants with a history of head injury, stroke, recurrent seizures, severe depression (Hamilton Depression Scale) (Estiasari et al., 2015), neurological deficits which may interfere with neurocognitive evaluation, pregnancy, breastfeeding and current use of illicit drugs were excluded. To establish a normative value, we recruited 82 local healthy controls similar in proportion of males (48% vs 68%), age (30 vs 31 years) and education (85% completed 9 years of education vs 78%) using the same criteria, plus no declared history of HIV risk behaviour. Subjects were assessed for pulmonary tuberculosis (TB), plasma HIV RNA was quantitated using COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Tests (version 2.0) and CD4 T-cell counts were determined using standard flow cytometric techniques. CD4 T-cells/μl and plasma HIV-RNA were assessed before ART and after 3, 6 and 12 months. The study was approved by the Faculty of Medicine Universitas Indonesia, Cipto Mangunkusumo National General Hospital, and Curtin University ethics committees.

2.2. Neurological assessments

All participants underwent baseline neurological assessment for five cognitive domains as per published methods (Estiasari et al., 2015, 2020). Follow up assessments were completed for HIV+ participants after receiving ART for 3, 6 and 12 months. Tests included Forward Digit Span to evaluate attention, Animal Naming Test to assess fluency, Rey Auditory Verbal Learning Test (immediate recall, delayed recall and learning over trials) to assess memory, Trail Making Test A and B to asses executive function, and Grooved Peg Board to assess motor speed. Z-scores for each domain were calculated by subtracting the neurocognitive test result from the mean normative value (obtained from the healthy controls) and dividing by the standard deviation of the normative value. The total cognitive Z-scores are an average of the Z-scores represented by each cognitive domain. Of the 82 participants, 21 participants did not complete follow-up clinical and neurocognitive assessments due to withdrawal from ART (n = 4), pregnancy (n = 2), death (n = 4), relocation (n = 4), and loss of contact (n = 7).

2.3. Genotyping

DNA samples were available for 59 of the 61 participants with complete neurological assessments. DNA samples were adjusted to 50 ng/μl and diluted 1:1 with TaqMan® OpenArray™ Genotyping Master Mix. Samples were genotyped for 20 SNP across *P2X7R* (Supplementary Table 1) using custom TaqMan® OpenArray™ Real-Time PCR Plates with the QuantStudio 12 K Flex Real-Time PCR System (Life Technologies, NY) and genotypes were assigned manually using the TaqMan® Genotyper Software and assessed as a dominant model (homozygous major allele versus hetero- or homozygous carriage of the minor allele). rs2230911 and rs3751144 were invariably co-inherited, so rs2230911 was excluded from analyses. Two SNP which failed to genotype in >10% of samples (rs208288 and rs1653609), two SNP that were monoallelic (rs10160951 and rs2230912), and three SNP that were carried by <10% of this group (rs1169737, rs17525767 and rs2857585) were excluded from analyses.

2.4. Statistical analyses

The effect of markers of HIV disease on neurocognitive Z-scores in HIV+ participants were assessed for each domain at 0 and 12 months on ART using Wilcoxon matched-pairs signed rank tests in GraphPad Prism version 8.2.1 for Windows (GraphPad Software, La Jolla, CA, USA). Multiple linear regression models were generated for Z-scores at all four time points, for each of the five cognitive domains and the total cognitive scores (Tables 2–5). Models included the SNP from *P2X7R* and demographic and clinical variables previously associated with neurocognitive outcomes; age, CD4 T-cells/μl and education (years) (Estiasari et al., 2020). Optimal models were determined using backward elimination of demographic and genetic predictors with $p > 0.1$ using the 'olsrr' v0.5.3 package (Hebbali, 2020) for the R programming language (Team, 2020). Models with a $p < 0.05$, an adjusted R-squared ≥ 0.1 and including one or more SNP are considered significant and discussed further (Tables 2–5). Regression coefficients are represented by Beta.

3. Results

3.1. Markers of HIV disease and cognitive function improved over 12 months on ART

Demographic, clinical and neurocognitive outcomes in this subset of 59 participants reflects the parent cohort, and demonstrate improvement of markers of HIV disease and neurocognitive function as described previously (Estiasari et al., 2015, 2020). The median (range) CD4 T-cell/μl and $\log_{10}$ of plasma HIV RNA/ml was 67 (2–199) and 5.15 (2.64–6.68) at baseline, and improved to 288 (44–763) and 1.30 (1.30–6.32), respectively, at 12 months on ART (Table 1), so ~60% of participants had undetectable plasma HIV RNA after 12 months of ART (Estiasari et al., 2020). TB was identified in 27/59 (46%) participants at baseline but did not influence Z-scores at baseline or at 12 months of ART ($p > 0.05$ for all cognitive domains; Supplementary Table 2). Z-scores improved between baseline and 12 months of ART ($p < 0.05$ for all cognitive domains; Table 1), with the exception of memory ($P = 0.67$; Table 1).

Table 1
Markers of HIV disease and neurocognitive outcomes improved over 12 months on ART.

	Time on ART				*0m vs 12m*
Variable[a]	0 months	3 months	6 months	12 months	*P*[b]
Age (years)	31 (19–48)				
Male Gender[c]	43 (73%)				
Education (years)	12 (6–16)				
TB co-infection[c]	27 (46%)				
CD4 T-cells/μl	67 (2–199)	189 (7–601)	208 (6–516)	288 (44–763)	<0.0001
Log_{10} HIV RNA/ml	5.15 (2.64–6.68)	1.67 (1.30–5.23)	1.30 (1.30–5.23)	1.30 (1.30–6.32)	<0.0001
Z-Attention	−0.78 (−2.44 to 1.81)	−0.53 (−1.94 to 2.23)	−0.36 (−1.94 to 1.81)	−0.11 (−1.94 to 2.23)	0.0004
Z-Fluency	−0.57 (−3.67 to 1.57)	−0.33 (−2.48 to 3.71)	−0.10 (−2.48 to 1.22)	−0.10 (−2.48 to 3.48)	<0.0001
Z-Memory	−2.06 (−6.92 to 1.22)	−2.46 (−6.92 to 1.22)	−2.49 (−6.96 to 1.16)	−2.72 (−6.19 to 1.25)	0.67
Z-Executive	0.26 (−5.94- to 1.29)	0.63 (−15.03 to 1.52)	0.77 (−3.00 to 1.50)	0.93 (−2.88 to 1.57)	<0.0001
Z-Motor Speed	−0.14 (−7.90 to 1.01)	0.21 (−8.88 to 1.43)	0.58 (−1.40 to 2.96)	0.65 (−2.82 to 1.75)	<0.0001
Z-Total Cognitive Function	−0.84 (−4.82 to 0.67)	−0.45 (−7.24 to 1.33)	−0.35 (−1.99 to 1.13)	−0.25 (−2.05 to 1.17)	<0.0001

[a] Median (range).
[b] Wilcoxon matched-pairs signed rank test between 0 and 12 months.
[c] n (%).

Table 2
SNP in *P2X7R* influence neurocognitive Z-scores prior to commencing ART.

Variable	Beta	95% CI[a]		*P*
		2.5%	97.5%	
Attention: Adjusted $R^2 = 0.112$ Model P = 0.030				
rs11065464	0.44	−0.002	0.89	0.05
rs504677	−1.03	−1.88	−1.18	0.02
rs1653598	1.13	0.26	2.00	0.01
Fluency: Adjusted $R^2 = 0.140$ Model P = 0.002				
Education	0.53	0.20	0.86	0.002
Memory: Adjusted $R^2 = 0.231$ Model P = 0.005				
rs208296	1.03	0.02	2.03	0.05
rs208307	2.62	1.01	4.24	0.002
rs503720	−2.20	−4.78	0.37	0.09
rs504677	−3.25	−5.42	−1.09	0.004
rs1653598	2.51	−0.07	5.08	0.06
Executive Function: Adjusted $R^2 = 0.310$ Model P = 0.0003				
Education	0.53	0.20	0.86	0.003
rs208307	0.78	−0.07	1.63	0.07
rs504677	−2.14	−3.22	−1.05	0.000
rs1653598	1.49	0.52	2.46	0.003
Motor Speed: Adjusted $R^2 = 0.260$ Model P = 0.0004				
Education	0.62	0.20	1.04	0.005
rs504677	−2.19	−3.39	−0.98	0.001
rs1653598	2.27	1.03	3.51	0.001
Total Cognitive: Adjusted $R^2 = 0.321$ Model P = 0.0002				
Education	0.39	0.12	0.67	0.006
rs208307	1.03	0.33	1.73	0.005
rs504677	−1.86	−2.76	−0.96	0.000
rs1653598	1.02	0.22	1.82	0.01

[a] CI; Confidence Interval.

Table 3
SNP in *P2X7R* influence neurocognitive Z-scores after 3 months on ART.

Variable	Beta	95% CI[a]		*P*
		2.5%	97.5%	
Attention: Adjusted $R^2 = 0.176$ Model P = 0.006				
Age	−0.06	−0.10	−0.02	0.007
rs208307	−0.58	−1.12	−0.04	0.04
rs3751144	−0.54	−1.08	−0.01	0.05
Fluency: Adjusted $R^2 = 0.136$ Model P = 0.002				
Education	0.20	0.07	0.32	0.002
Memory: Adjusted $R^2 = 0.193$ Model P = 0.005				
Age	−0.10	−0.18	−0.03	0.008
rs1718125	1.07	1.04	2.04	0.03
rs208296	0.87	−0.05	1.80	0.06
rs12301635	−1.33	−2.49	−0.18	0.03
Executive Function: Adjusted $R^2 = 0.433$ Model P = 0.0000				
Age	0.07	−0.01	0.16	0.08
Education	0.31	0.12	0.50	0.002
rs208307	2.75	1.04	4.45	0.002
rs504677	−6.37	−8.59	−4.16	0.000
rs1653598	3.89	1.95	5.82	0.000
Motor Speed: Adjusted $R^2 = 0.334$ Model P = 0.0001				
Education	0.15	0.04	0.26	0.07
rs208307	1.05	0.09	2.01	0.03
rs504677	−2.84	−4.06	−1.61	0.000
rs1653598	1.81	0.71	2.91	0.002
Total Cognitive: Adjusted $R^2 = 0.324$ Model P = 0.0002				
Education	0.16	0.02	0.30	0.03
rs208307	1.54	0.26	2.83	0.02
rs504677	−3.85	−5.49	−2.21	0.000
rs1653598	2.69	1.22	4.16	0.001

[a] CI; Confidence Interval.

3.2. Linear regression models identified SNP from P2X7R as predictors of neurocognitive outcomes in all domains, except fluency

Linear regression models for the Z-scores of the five neurocognitive domains and total cognitive function at 0, 3, 6 and 12 months of ART were determined using backward elimination of covariables with $p > 0.1$ (Tables 2–5). Optimal models achieving analyses criteria of an adjusted $R^2 > 0.1$, model $p < 0.05$ and inclusion of ≥1 SNP were identified for all domains except fluency and are presented separately for each time point (Tables 2–5). Outcomes for each domain are described below.

Attention: Three SNP (rs504677, rs11065464 and rs1653598) remained in the optimal model before ART (Table 2), with rs1653598 remaining at 12 months (P = 0.006). Carriage of the minor allele of rs1653598 associated with positive attention outcomes at baseline and at 12 months (Tables 2 and 5). Attention also associated with rs10849849 and rs1718125 at 12 months (P = 0.001 and 0.005, respectively; Table 5).

Fluency: Models from all time points achieved an adjusted $R^2 > 0.1$ with $P < 0.001$, but none retained any *P2X7R* SNP. Fluency was consistently influenced by level of education (Tables 2–5).

Memory: The optimal model for memory outcomes before ART had an adjusted R^2 of 0.231 (P = 0.005) and included five SNP, of which rs208296, rs208307 and rs504677 were significantly associated with memory Z-scores ($P < 0.05$; Table 2). rs208296 remained in the optimal memory model at 3 months (P = 0.06; Table 3), and associated with

Table 4
SNP in *P2X7R* influence neurocognitive Z-scores after 6 months on ART.

Variable	Beta	95% CI[a]		*P*
		2.5%	97.5%	
Attention: Adjusted $R^2 = 0.026$ Model P = 0.117				
Education	0.06	0.00	0.14	0.12
Fluency: Adjusted $R^2 = 0.237$ Model P = 0.0002				
Age	−0.06	−0.11	−0.01	0.03
Education	0.22	0.10	0.34	0.001
Memory: Adjusted $R^2 = 0.202$ Model P = 0.002				
Age	−0.14	−0.21	−0.06	0.000
rs208296	0.94	0.00	1.88	0.05
rs1653598	−0.85	−1.78	0.09	0.07
Executive Function: Adjusted $R^2 = 0.221$ Model P = 0.0013				
Education	0.10	0.05	0.16	0.001
rs504677	−0.70	−1.27	−0.13	0.02
rs1653598	0.67	0.09	1.26	0.03
Motor Speed: Adjusted $R^2 = 0.135$ Model P = 0.016				
Age	−0.03	−0.06	−0.002	0.04
rs504677	−0.74	−1.42	−0.06	0.03
rs1653598	0.64	−0.06	1.33	0.07
Total Cognitive: Adjusted $R^2 = 0.320$ Model P = 0.0001				
Age	−0.05	−0.08	−0.02	0.001
Education	0.10	0.04	0.17	0.004
rs208296	0.33	−0.04	0.71	0.08
rs504677	−0.33	−0.70	0.04	0.08

[a] CI; Confidence Interval.

Table 5
SNP in *P2X7R* influence neurocognitive Z-scores after 12 months on ART.

Variable	Beta	95% CI[a]		*P*
		2.5%	97.5%	
Attention: Adjusted $R^2 = 0.182$ Model P = 0.009				
rs10849849	1.52	0.70	2.34	0.001
rs1718125	−1.08	−1.82	−0.34	0.005
rs11065464	0.46	−0.03	0.94	0.06
rs1653598	0.91	0.28	1.55	0.006
Fluency: Adjusted $R^2 = 0.137$ Model P = 0.002				
Education	0.67	0.52	1.10	0.002
Memory: Adjusted $R^2 = 0.058$ Model P = 0.087				
rs208307	1.71	0.13	3.28	0.03
rs504677	−1.61	−3.18	−0.03	0.05
Executive Function: Adjusted $R^2 = 0.277$ Model P = 0.007				
rs10849849	0.48	0.03	0.93	0.04
rs208307	0.75	0.12	1.39	0.02
rs504677	−1.03	−1.69	−0.36	0.003
rs3751144	−0.64	−1.11	−0.16	0.009
Motor Speed: Adjusted $R^2 = 0.129$ Model P = 0.010				
CD4 T-cells/μL	−0.001	−0.003	0.00	0.03
rs504677	−0.44	−0.86	−0.02	0.04
Total Cognitive: Adjusted $R^2 = 0.321$ Model P = 0.0002				
Age	−0.03	−0.06	−0.004	0.02
rs504677	−0.32	−0.67	0.04	0.08

[a] CI; Confidence Interval.

memory at 6 months (P = 0.05; Table 4). The rs208296 minor allele had a positive effect on memory outcomes at 0, 3 and 6 months (Tables 2–4). At 12 months, rs208307 and rs504677 were significantly associated with memory, but the model did not meet our analyses criteria (Table 5).

Executive function: Robust models were identified for executive function at 0, 3, 6 and 12 months after ART (Adjusted $R^2 = 0.22$ to 0.43, P = 0.007 to 0.0000; Tables 2–5). Three SNP (rs504677, rs208307 and rs1653598) consistently influenced executive outcomes after adjusting for education at 0, 3 and 6 months on ART and for age at 3 months on ART. Carriage of the minor allele of rs504677 associated with lower Z-scores at all four time points. Optimal models at baseline and 3 months retained rs208307 and rs1653598, at 6 months included rs1653598 and at 12 months included rs208307 (Tables 2–5).

Motor speed: The optimal model at baseline, 3 and 6 months included rs504677 and rs1653598 after adjusting for education (0 and 3 months; Tables 2 and 3) or age (6 months; Table 4). At 12 months, only rs504677 remained in the optimal model after adjustment for CD4 T-cell counts. The effects of age and CD4 T-cells/μl on motor speed Z-scores were marginal and rs504677 was consistently linked with lower Z-scores, as noted with other domains.

Total cognitive function: Optimal models at each time point reflected models for each domain at the corresponding time point. All included *P2X7R* SNP after adjusting for education (0, 3 and 6 months; Tables 2–4) and/or age (6 and 12 months; Tables 4 and 5). Accordingly, carriage of the minor allele of rs504677 is independently associated with lower Z-scores at baseline (P = 0.0006; Table 2) and at 3 months after ART ($p < 0.001$; Table 3), and remained in the optimal models at 6 and 12 months but did not reach significance (P = 0.08 and 0.08; Tables 4 and 5).

4. Discussion

This study provides unique insights into neurocognitive function across five domains in HIV+ Indonesians prior to commencing ART and at 3, 6 and 12 months of ART. Furthermore, we assessed associations between *P2X7R* SNP and the neurocognitive outcomes at these time points. This creates a large number of potential associations, and the longitudinal design complicates corrections for multiple comparisons. We address this by confining the discussion to associations evident in multiple domains or more than one timepoint. We report improvements of neurocognitive outcomes and associations with *P2X7R* SNP that vary by domain and time on ART.

By following patients responding to ART, we also shed light on the process of recovery of neurocognitive capacity. Patients began ART with <200 CD4 T-cells/μl, so they were experiencing a range of inflammatory symptoms affecting their general health and neurocognitive performance. With the exception of the memory domain, all Z-scores improved over time to approximate the healthy controls after 6–12 months (Estiasari et al., 2020), but rates differed by domain. It is therefore plausible that anti-inflammatory mechanisms were activated differentially or as a cascade. Rubin et al. demonstrated that levels of microglial activation varied between brain regions of virally-suppressed HIV+ individuals, and higher levels of microglial activation are inversely associated with neurocognitive outcomes in a domain-specific manner (Rubin et al., 2018). Additionally, immune responses can be affected by suboptimal adherence or changes to ART (García de Olalla et al., 2002). Short periods of non-adherence to ART have been associated with memory deficits (Obermeit et al., 2015). This may complicate longitudinal analyses. It is pertinent here that only ~60% of participants were virally suppressed after 12 months and several had changed their treatment regimens.

Memory was the only domain that did not improve over 12 months of ART (Table 1). Memory deficits have been described in individuals with viral suppression through effective ART (Rubin et al., 2018; Heaton et al., 2010b), but may also reflect the severity of HIV disease before treatment (Tozzi et al., 2007; Ellis et al., 2011). Participants in our study commenced ART with only 67 (2–199) CD4 T-cell/μl - which may enhance persistent neurological defects. Furthermore, the JakCCANDO cohort has a high burden of cytomegalovirus (CMV). CMV was associated with more severe HIV disease in a Thai population (Durier et al., 2013) and with neurocognitive impairment in older adults without HIV (Luz Correa et al., 2014). We associated the baseline burden of CMV with

memory and total cognitive outcomes after 6 months on ART in this population (Estiasari et al., 2020).

No alleles of *P2X7R* associated with fluency scores and optimal models consistently associated fluency with education (Tables 2–5). The cohort included individuals with only primary education and several who had completed university. The socioeconomic sequelae of this range in a large Asian city (Jakarta) provides further scope to identify an effect as "education". Older age has been associated with poorer fluency outcomes (Elgamal et al., 2011) but the cohort is relatively young and uniform in age [31 (19–48) years].

When we commenced this study, we anticipated associations with coding SNP which impact the function of P2X7R and therefore influence inflammation. We included six exonic SNP, but only two met our inclusion criteria for bivariate and multivariable analyses (Supplementary Table 1). One of the SNP excluded was rs2230912, a missense variant associated with neuropsychological disorders (McQuillin et al., 2009; Erhardt et al., 2007), as the minor allele was not detected in this population. This highlights differences in patterns of LD between ethnicities and may suggest P2X7R pathways differ between disease phenotypes. Our results instead identified five intronic SNP which influence neurocognitive outcomes in a time- and domain-specific manner in HIV+ Indonesians as they recover on ART. These will be discussed individually.

4.1. rs504677

rs504677 associated with lower Z-scores across all domains (except fluency) and at all four timepoints in executive function, motor speed and total cognitive function (Tables 2–5). rs504677 has a RegulomeDB score of 2b (https://regulome.stanford.edu), suggesting this SNP affects transcription factor binding and therefore may influence neurocognitive outcomes via the regulation of expression and splicing of *P2X7R* (Boyle et al., 2012). This variant has been linked to altered expression and splice variants of both *P2X7R* and the neighbouring gene encoding purinergic P2X receptor 4 (*P2X4R*) in the Gene Tissue Expression (GTEx) Portal (https://gtexportal.org/; accessed Nov 2020). Furthermore, rs504677 is in LD (r^2 and D' >0.90) with more than 25 intronic *P2X7R* SNP within a ±5000 base pair region in the 1000 Genomes East Asian (EAS) populations, and so may also mark a causal variant outside our genotyping panel (https://ldlink.nci.nih.gov/).

4.2. rs1653598

rs1653598 occurs in all optimal models prior to commencing ART (excluding fluency), with executive and motor speed domains at 3 and 6 months, memory at 6 months, and attention at 12 months (Tables 2–5). Carriage of the minor allele of rs1653598 is consistently linked to higher Z-scores. As with rs504677, this allele is linked with altered expression and splicing of *P2X7R* and *P2X4R* (https://gtexportal.org/), and is in LD with over 25 *P2X7R* SNP in the EAS population in a window ±5000 base pairs. One SNP in LD ($r^2 = 1.0$, D' = 1.0) is a gain-of-function variant, rs1718119 (https://ldlink.nci.nih.gov/). rs1718119 is associated with higher pain intensity scores in females with diabetic peripheral neuropathy (Ursu et al., 2014) and with inflammatory conditions including systemic lupus erythematosus, chronic obstructive pulmonary disease, and localised aggressive periodontitis (Chen et al., 2013; Dai et al., 2018; Harris et al., 2020). Homozygous carriage of the minor allele of rs1718119 is associated with an ATP-induced increase of IL-1β from monocytes and with increased levels of IL-6 in whole blood (Harris et al., 2020; Stokes et al., 2010). This fits with a role in HAND and warrants investigation.

4.3. rs11065464

The rs11065464 minor allele was explicitly linked with modest improvements in attention Z-scores at baseline and after treatment with ART for 12 months (Tables 2 and 5). This allele is linked in the GTEx Portal with altered expression of *P2X4R* and with altered splicing of calcium/calmodulin-dependent kinase kinase 2 (*CAMKK2*) and *P2X7R* (https://gtexportal.org/) but evidence of a pathological role for this SNP is lacking.

4.4. rs208296

Carriage of the minor allele of rs208296 is consistently associated with higher memory Z-scores in the optimal models at 0, 3 and 6 months (Tables 2–4). rs208296 has a regulomeDB score of 1f (https://regulome.stanford.edu) indicating a highly regulatory role, and is associated with altered expression of *P2X4R* and splicing of *CAMKK2* (https://gtexportal.org/). Carriage of the minor allele correlates with increased cold pain tolerance in a Finnish population (Kambur et al., 2018) suggesting a neurological role for this SNP. However, no studies assess its role in inflammation or memory/cognition.

4.5. rs208307

rs208307 associated with higher Z-scores in memory and executive domain optimal models at multiple timepoints. This allele was linked with HIV-SN in Africans treated with stavudine but not in Africans and Indonesians treated without stavudine (Gaff et al., 2019b, 2020; Goullee et al., 2016). rs208307 is located at an acceptor splice site in intron 6 and is associated with altered expression and splicing of *P2X7R* in the GTEx Portal (https://gtexportal.org/). Furthermore, carriage of the rs208307 minor allele associated with higher levels of P2X7R mRNA lacking exons 7 and 8, which is predicted to impair P2X7R function (Skarratt et al., 2020). It is plausible that associations between memory and executive Z-scores and rs208307 are mediated by higher levels of exon 7 and 8 skipping in P2X7R mRNA and warrants investigations. We were able to replicate our finding in Australian HIV patients (Gott et al., 2017). These were males (n = 49) aged 57 (45–73) years, of European descent and tested after >2 years on ART. We associated carriage of the minor allele with higher scores for verbal learning [49 (18–66) vs 41 (10–61), Mann Whitney P = 0.04] and verbal memory [50 (23–61) vs 40 (10–63), P = 0.009] (unpublished data).

Overall, this study provides novel insights in time- and domain-specific neurocognitive changes in HIV+ Indonesians over the first 12 months on ART. We identify five intronic polymorphisms which associate with neurocognitive outcomes in specific domains. These may influence the levels or isoforms of P2X7R expressed, or may be in LD with causal SNP. P2X7R is abundantly expressed in microglia and overexpression of P2X7R drives microglial activation (Monif et al., 2016; Chen et al., 2016). Microglial activation differs between regions of the brain in HIV+ individuals and correlates with domain-specific cognitive impairments, so it is plausible that our SNP may impact HIV-associated neurocognitive outcomes. Further genetic investigations in larger cohorts of defined ethnicity are warranted.

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Declaration of competing interest

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

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Supplementary Table 1. DNA samples were genotyped for 20 *P2X7R* SNP and 12 were included in bivariate and multivariable analyses

#	SNP ID	Minor/Major[a]	MAF[b]	Transcript	Included in Analyses	
1	rs10849849	G/A	0.27	Intronic	Yes	
2	rs208288[c]	C/G	0.06	Intronic	No	Genotyping Failure
3	rs17525767	T/C	0.05	Intronic	No	<10% Carriage
4	rs1718125	T/C	0.37	Intronic	Yes	
5	rs1169737	T/C	0.03	Exonic	No	<10% Carriage
6	rs1186055	A/C	0.33	Intronic	Yes	
7	rs2857585	A/G	0.05	Intronic	No	<10% Carriage
8	rs208296	A/G	0.34	Intronic	Yes	
9	rs11065464	A/C	0.25	Intronic	Yes	
10	rs208307	G/C	0.22	Intronic	Yes	
11	rs503720	A/G	0.19	Intronic	Yes	
12	rs504677	T/C	0.22	Intronic	Yes	
13	rs1653609	A/C	0.44	Intronic	No	Genotyping Failure
14	rs2230911	G/C	0.24	Exonic	No	LD with rs3751144
15	rs1653598	C/T	0.19	Intronic	Yes	
16	rs10160951	G/C	0.01	Exonic	No	Monoallelic
17	rs2230912	G/C	0.00	Exonic	No	Monoallelic
18	rs3751144	T/C	0.26	Exonic	Yes	
19	rs3751143	C/A	0.20	Exonic	Yes	
20	rs12301635	G/C	0.12	3’ UTR	Yes	

[a] Minor and Major alleles as determined in this population

[b] MAF; Minor allele frequency in the 59 participants in this study

[c] SNP excluded from analyses are shaded

Table 2. TB does not influence neurocognitive Z-scores at baseline or after 12 months of ART

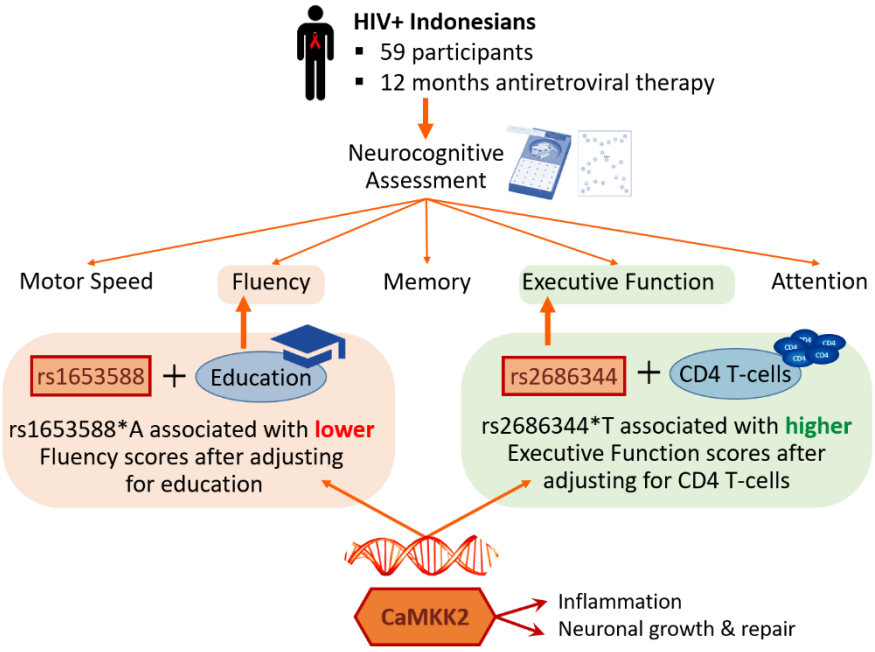
Domain Z-scores	TB +ve n=27/59 (46%)	TB -ve n=32/59 (54%)	P[a]
Baseline			
Attention	-0.86 (-2.44 to 0.98)	-0.78 (-2.11 to 1.81)	0.98
Fluency	-0.81 (-3.67 to 1.10)	-0.33 (-2.48 to 1.57)	0.14
Memory	-2.94 (-6.92 to 0.81)	-1.96 (-6.46 to 1.22)	0.40
Executive	-0.12 (-5.42 to 1.29)	0.48 (-5.94 to 1.08)	0.27
Motor Speed	-0.42 (-7.90 to 1.01)	-0.07 (-3.38 to 0.99)	0.26
Total	-0.89 (-4.82 to 0.57)	-0.77 (-2.50 to 0.67)	0.28
12 months of ART			
Attention	-0.11 (-1.94 to 2.23)	-0.11 (-1.61 to 1.14)	0.42
Fluency	-0.81 (-1.76 to 3.48)	0.38 (-2.48 to 3.24)	0.19
Memory	-2.41 (-6.19 to 1.25)	-2.78 (-5.71 to 0.70)	0.83
Executive	0.90 (-1.16 to 1.57)	1.00 (-2.88 to 1.42)	0.36
Motor Speed	0.53 (-2.02 to 1.75)	0.76 (-2.82 to 1.50)	0.92
Total	-0.52 (-2.05 to 1.17)	-0.18 (-1.57 to 0.96)	0.46

[a] Wilcoxon rank sum test. Data are presented as Median (range).

Chapter 8

Polymorphisms in *CAMKK2* may influence domain-specific neurocognitive function in HIV+ Indonesians receiving ART

In Chapter 7, I identified domain and time-specific associations between P2X7R polymorphisms and neurocognitive outcomes in HIV+ Indonesians for the first 12 months receiving ART excluding stavudine. As the P2X4R and CAMKK2 genes are adjacent to P2X7R in a region of linkage disequilibrium, I considered whether P2X4R and CAMKK2 may also impact neurocognitive outcomes in HAND in HIV+ Indonesians who had received ART for 12 months.



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Polymorphisms in *CAMKK2* may influence domain-specific neurocognitive function in HIV+ Indonesians receiving ART

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Running Title:

CAMKK2 and neurocognition in HIV patients

ABSTRACT

Background: Despite effective antiretroviral therapy (ART), milder forms of HIV-associated neurocognitive disorders (HAND) remain prevalent, and are characterised by neuroinflammation, synaptic dysfunction and neuronal loss.

Methods: We explore associations between neurocognitive impairment in HIV+ Indonesians and 17 polymorphisms in neighbouring genes involved in inflammation and neuronal growth/repair pathways, *P2X4R* and *CAMKK2*. HIV+ Indonesians (n=59) who had received ART for 12 months were assessed to derive Z-scores for the attention, fluency, memory, executive and motor speed domains relative to local control subjects. These were used to determine total cognitive scores.

Results: No alleles of *P2X4R* displayed significant associations with neurocognition in bivariate or multivariable analyses. In *CAMKK2*, rs2686344 influenced total cognitive scores in bivariate analyses (p=0.04). Multivariable linear regression modelling independently associated rs2686344 with higher executive function Z-scores (P=0.05) after adjusting for CD4 T-cell counts (Adjusted R^2=0.103, Model P=0.034), whilst rs1653588 associated with lower and rs1718120 (p=0.05) with higher fluency Z-scores (p=0.05) after adjusting for education and $\log_{10}$ HIV RNA copies/ml (Adjusted R2=0.268, Model P=0.001).

Conclusions: Polymorphisms in *CAMKK2* may influence neurocognitive outcomes in specific domains in HIV+ Indonesians receiving ART for 12 months.

Words: 183

Keywords

HIV, neurocognitive impairment, *P2X4R* and *CAMKK2*, single nucleotide polymorphisms, Indonesia

INTRODUCTION

Despite effective antiretroviral therapy (ART), milder forms of HIV-associated neurocognitive disorders (HAND) remain prevalent [1, 2] and can impact ability to work, quality of life, and adherence to ART. Around 50% of our cohort of HIV+ Indonesians beginning ART with <200 CD4 T-cells/µl experienced HAND [1, 3]. Neurocognitive function improved over 6 months at rates influenced by age, education and/or CD4 T-cell counts, but memory function remained poor [1, 3]. Neuroinflammation, synaptic dysfunction and neuronal degeneration are hallmarks of HAND [4]. Accordingly, markers of microglial activation and synaptic dysfunction are associated with neurocognitive impairment in HIV+ individuals [5, 6]. Purinergic P2X receptor 4 (P2X4R) and calcium/calmodulin-dependent kinase kinase 2 (CaMKK2) are involved in neuroinflammatory and neuronal growth and repair pathways [7, 8] and so may play a role in HAND.

P2X4R are ATP-gated cation channel proteins found abundantly in the central nervous system and highly expressed by microglia [7]. Stimulation of P2X4R drives an influx of calcium ions and activation of p38 mitogen-activated protein kinase (MAPK) [9]. In microglia, p38 MAPK signalling regulates expression of cytokines including interleukin 1-beta (IL-1β) and tumour necrosis factor-alpha (TNFα) [10]. This can promote synaptic and neuronal loss in co-cultured microglia and neurons treated with lipopolysaccharide [10]. Additionally, cultured satellite glial cells from rat dorsal root ganglia treated with HIV envelope glycoprotein 120 exhibited decreased viability correlated with increased phosphorylation of p38 MAPK and production of IL-1β. These increases were attenuated by inhibition of P2X4R [11].

CaMKK2 activates the AMP-activated protein kinase [8] which activates p38 MAPK pathways [12] and so has potential to elicit a cytokine response. However CaMKK2 has a clearer neurological role, involved in synapse and dendrite development, axonal growth and repair, neuronal survival, and learning and memory formation [8]. In cultured hippocampal neurons, CaMKK2 phosphorylates calcium/calmodulin-dependent kinase I (CaMKI), which phosphorylates and complexes with βPak-interacting exchange factor. In turn, this activates Rac1, a member of the Rho family GTPases which stimulates spine and synapse formation [13]. Inhibition of CaMKK2 or CaMKI decreased spine formation, and silencing of CaMKK2 impaired maturation of spines - thus inhibiting synapse formation [13].

P2X4R and *CAMKK2* are adjacent genes in a region of high linkage disequilibrium [14]. We have associated polymorphisms in *P2X4R* and *CAMKK2* with altered concentrations of TNFα *in vitro* [15], and with HIV-associated sensory neuropathy (HIV-SN) affecting peripheral nerves [14, 16, 17]. SNPs were selected based on (in order) exonic location, published links with inflammatory/neurological diseases, location in proximal untranslated regions and presence in more than one population. A *CAMKK2* intronic SNP, rs1063843, was associated with decreased expression of CaMKK2 in the dorsolateral prefrontal cortex, deficits in working memory and executive function, and increased risk of schizophrenia [18]. Here we use multivariable

regression models to address the hypothesis that associations between polymorphisms in *P2X4R* and *CAMKK2* influence cognitive function in HIV+ Indonesians treated with ART for 12 months. As HIV does not affect neurocognitive domains equally, this may illuminate which pathways are impacted by *P2X4R* and *CAMKK2*.

MATERIALS AND METHODS

82 HIV+ Indonesians were recruited at Cipto Mangunkusumo National General Hospital in Jakarta, Indonesia [1, 3]. Participants were ART-naïve, had <200 CD4 T-cells/µl, a Karnofsky performance score of 70-100 and were living in Jakarta. Exclusion criteria included history of recurrent seizures, severe depression, stroke, head injury, neurological deficits which may interfere with neurocognitive evaluation, pregnancy, breastfeeding and current use of illicit drugs. An additional 82 age-, gender- and education-matched local healthy controls were recruited with the same criteria plus no declared HIV risk behaviour. Patients were assessed for pulmonary tuberculosis (TB), plasma HIV RNA was quantitated using COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Tests (version 2.0) and CD4 T-cell counts were determined using standard flow cytometric techniques. All participants provided written and informed consent, and the study was approved by the Human Research Ethics Committee of the Faculty of Medicine Universitas Indonesia, Cipto Mangunkusumo National General Hospital and Curtin University.

Neurocognitive assessments over five domains [19] were completed at baseline and after 12 months on ART for HIV+ participants, and once for healthy controls to establish demographically adjusted normative values [1, 3]. Briefly, attention was assessed using the Forward Digit Span, fluency by the Animal Naming Test, memory by the Rey Auditory Verbal Learning Test (immediate recall, delayed recall and learning over trials), executive function by the Trail Making Test A and B, and motor speed by the Grooved Peg Board test. The test results were subtracted from the mean normative value and then divided by the standard deviation of the normative value to calculate Z-scores for each domain. The average of the Z-scores for each domain were calculated to derive the total cognitive function Z-scores. Of the 82 participants, 61 completed baseline and follow-up neurocognitive assessment after 12 months of ART. DNA samples were available for 59 of the 61 participants for genotype analyses.

The 59 DNA samples were diluted to 50ng/µl and mixed with TaqMan® OpenArray™ Genotyping Master Mix at a 1:1 ratio. Samples were genotyped for 17 SNP across *P2X4R* and *CAMKK2* with custom TaqMan® OpenArray™ Real-Time PCR Plates on the QuantStudio 12K Flex Real-Time PCR System (Life Technologies, NY). Genotypes were determined manually using the TaqMan® Genotyper Software. Four SNP from *P2X4R* (rs2303998, rs10849860, rs11608486 and rs7961979) and three SNP from *CAMKK2* (rs11065502, rs3714454 and rs3817190) were carried by less than <10% of participants or failed to genotype in more than 10% of

samples and so were excluded from analyses. *P2X4R* (rs25643) and *CAMKK2* (rs1560568) were excluded as they were co-inherited with tested SNP (rs7298368 and rs7975295, respectively).

Z-scores of individuals carrying one or two copies of the minor alleles were compared to Z-scores of individuals with homozygous carriage of the major allele (dominant models; Supplementary Table 1) with Wilcoxon rank sum tests in the R statistical programming environment (v1.3.959) [20]. No corrections were made for multiple comparisons. Linear regression modelling was completed using the 'olsrr' v0.5.3 package for R [21, 22] and included variables previously associated with neurocognitive scores [age, CD4 T-cells/µl, years of education [1]], $\log_{10}$ HIV RNA copies/ml, plus SNP from *P2X4R* or *CAMKK2*. Optimal models were determined for each domain using backward elimination until only variables achieving $p<0.1$ remained [22]. Only models achieving an adjusted R-squared (R^2) ≥0.1, a model $p<0.05$, and with at least one SNP are discussed.

RESULTS AND DISCUSSION

The demographic, clinical and neurocognitive outcomes in this subset of 59 participants reflects the parent cohort described previously [1, 3]. The median (range) age was 31 (19-48) years, education completed was 12 (6-16) years, CD4 T-cell counts were 288 (44-763) cells/µl, and $\log_{10}$ plasma HIV RNA was 1.30 (1.30-6.32) copies/ml after 12 months on ART (Supplementary Table 2). Pulmonary TB was identified in 27/59 (46%) participants at baseline but did not influence Z-scores after 12 months of ART (P-values = 0.20 to 0.92; Supplementary Table 2).

P2X4R SNP were not retained in linear regression models

No alleles of *P2X4R* achieved $P<0.05$ in bivariate analyses for any domain or total cognitive function after 12 months of ART (Table 1). Optimal linear regression models were based on age, CD4 T-cell counts, Log_{10} HIV RNA copies/ml and education, and did not retain either SNP (Supplementary Table 3). This suggests a limited role for *P2X4R* in HAND. However, we only tested two SNP and it is pertinent that animal and *in vitro* studies suggest a role for P2X4R in neuropathic pain, memory loss and anxiety, and synaptogenesis [23-25]. We were able to validate the effect of rs7298368 in Australian HIV patients [26]. These were males (n=72), aged 55(42-74) years and of European descent, and had been on ART for over 2 years [26]. We identified significant effects on mean T-scores for mental flexibility [51.1 (29-78) *vs* 47.9(24-68), p=0.01] and verbal fluency [54.1 (33-69) *vs* 47.7(36-64), p=0.03] (unpublished data). Genetic studies of other *P2X4R* SNP in larger cohorts should consider ethnicity, as we have shown that this has clear effects on linkage disequilibrium influencing the haplotypes that predominate in the populations [14, 16, 17].

Fluency and executive function domain Z-scores were influenced by SNP in *CAMKK2*

In bivariate analyses, five *CAMKK2* SNP achieved P=0.06-0.18 in at least one of the five domains or total cognitive function. rs2686344 associated significantly with total cognitive function (P=0.04; Table 1). Of the five, rs11065504 and rs7975295 associated with reduced risk of HIV-SN in Africans [17] and Indonesians [16] treated without stavudine (respectively).

Models for two domains retained *CAMKK2* SNP and achieved an Adjusted R^2>0.1 and model P<0.05 (Table 2). Those failing these criteria are presented but are not discussed further. The optimal model for fluency (Adjusted R^2=0.268, P=0.001) included education, $\log_{10}$ HIV RNA copies/ml, rs1653588 and rs1718120. The minor allele of rs1653588 associated with poor fluency (P=0.04; Table 2) and the minor allele of rs1718120 associated with higher fluency Z-scores (P=0.05; Table 2). rs1653588 is a non-coding variant located in the 3-prime untranslated region of *CAMKK2*, so carriage of this allele may impact neurocognitive function via altered expression of *CAMKK2* or neighbouring genes. The Gene Tissue Expression (GTEx) Portal (version 8) is an online reference resource which reports associations between gene expression levels and genotype in non-diseased human tissue. The GTEx portal associates rs1653588*A with altered *P2X4R* expression and splice variants (https://gtexportal.org/; accessed Nov 2020).

The optimal model for executive function retained rs2686344 and rs1718120 after adjusting for CD4 T-cell counts (Table 2). Carriage of the minor alleles of both SNP was linked with higher Z-scores. rs2686344 is an intronic variant associated with altered *CAMKK2* expression in the GTEx Portal (https://gtexportal.org/), but yields conflicting results. Here it approached significant associations with fluency and total cognitive Z-scores and not executive function in bivariate analyses (Table 1), so interactions with clinical factors may be important. rs2686344 associated with lower rates of peripheral neuropathy in Africans treated with stavudine, but showed no effect in Africans and Indonesians treated without stavudine [14, 16, 17]. Stavudine probably inhibits mitochondrial function in peripheral nerves, so the ART regimen may be important. We sought validation of associations between rs2686344 and cognitive deficits in the Australian HIV patients as described above. These individuals were tested in 2009-11 so some had used stavudine or related drugs at some time [26]. Carriage of the minor allele associated with higher T-scores for speed information processing [51.9(42-66) vs 47.6 (17-69), P=0.038], with a marginal effect on verbal memory [44 (10-63) vs 49.5(23-61), P=0.11] (unpublished data).

rs1718120 displayed weak links with Z-scores in all five domains in bivariate analyses (Table 1) and was included in the optimal models for fluency and executive function (Table 2). The association with carriage of the minor allele in the optimal models was positive but weak (0.05 < P < 0.10). rs1718120 is intronic and has been associated with altered expression of *CAMKK2* and the upstream gene *ANAPC5* (https://gtexportal.org/). We associated SNP in *ANAPC5* with large fibre neuropathy in HIV+ Indonesians [27].

ANAPC5 encodes a subunit of the anaphase promoting complex (APC) which initiates cell progression from metaphase into anaphase. Replication of neurons would disturb signal transmission, so aberrant re-entry into the cell cycle elicits apoptosis [28]. Accordingly, Almeida et al demonstrated that dysregulation of APC pathways in cultured primary rat cortical neurons triggers apoptotic neuronal death [28].

The exploratory nature of this study and the modest number of participants limited our ability to assess rare SNP and correct for multiple comparisons. Nonetheless, our study identifies SNP in *CAMKK2* which may contribute to HIV-associated neurocognitive impairment in specific domains. Overall, these results suggest a role for *CAMKK2* in neurocognitive impairment in HIV+ individuals and warrants further investigation.

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Conflict of Interest

The authors declare that they have no conflict of interest

Data Availability

Data are available on request.

Author contributions

JG conducted statistical analyses and wrote the manuscript, RE designed and conducted the neurocognative assessments, DD assisted with neurocognative assessments, SH performed the genotyping, PK provided statistical advice, PP co-ordinated the project.

Abbreviations

ART	Antiretroviral therapy
CAMKI	Calcium/calmodulin-dependent kinase I
CaMKK2	Calcium/calmodulin-dependent kinase kinase 2
GTEx	Gene Tissue Expression

HAND	HIV-associated neurocognitive disorder
HIV-SN	HIV-associated sensory neuropathy
IL-1β	Interleukin 1-beta
MAPK	Mitogen-activated protein kinase
P2X4R	Purinergic P2X receptor 4
TB	Tuberculosis
TNFα	Tumour necrosis factor-alpha

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Table 1. rs2686344 in *CAMKK2* associated with total cognitive function in HIV+ Indonesians after receiving ART for 12 months

		Cognitive domain					
	MAF [b]	**Attention**	**Fluency**	**Memory**	**Executive**	**Motor Speed**	**Total**
Z-Scores	**-**	-0.11	-0.10	-2.72	0.93	0.65	-0.25
Median (range)		(-1.94 to 2.23)	(-2.48 to 3.48)	(-6.19 to 1.25)	(-2.88 to 1.57)	(-2.82 to 1.75)	(-2.05 to 1.17)
		p-values for Mann-Whitney U tests [a]					
***P2X4R* SNP ID**							
minor/major allele							
rs2686387 G/C	0.43	**0.20**[c]	0.47	0.90	0.43	0.27	0.99
rs7298368 T/C	0.45	0.43	0.56	0.79	0.87	**0.09**	0.88
***CAMKK2* SNP ID**							
minor/major allele							
rs1653587 T/A	0.09	0.38	0.48	0.79	0.62	0.52	0.54
rs1653588 A/T	0.08	0.77	**0.11**	0.85	0.87	0.79	**0.15**
rs11065504 C/G	0.32	0.27	0.80	0.28	0.71	**0.18**	0.83
rs7975295 C/T	0.32	0.92	0.83	**0.17**	**0.16**	0.97	0.39
rs2686344 T/C	0.24	0.93	**0.06**	0.23	0.21	1.00	**0.04**[d]
rs1718120 G/T	0.26	**0.10**	**0.12**	**0.17**	**0.12**	**0.12**	0.45

[a] Dominant model; Heterozygous or homozygous minor allele versus homozygous major allele;

[b] MAF; Minor allele frequency;

[c] Variables achieving P<0.20 are bolded;

[d] Variables achieving P<0.05 are underlined

Table 2. SNP in *CAMKK2* differentially influence neurocognitive outcomes after 12 months of ART

Variable	β [b]	95% CI [a]		*P*
		2.5%	97.5%	
Attention: Adjusted R^2=0.050, Model P=0.054				
rs1718120	0.47	-0.01	0.94	0.05
Fluency: Adjusted R^2=0.268, Model P=0.001 [c]				
Education	0.20	0.08	0.32	0.002
HIV RNA copies/ml	-0.24	0.05	0.29	0.10
rs1653598	-0.93	-1.79	-0.06	0.04
rs1718120	0.65	-0.01	1.32	0.05
Memory: Adjusted R^2=0.103, Model P=0.018				
Age	-0.09	-0.16	-0.02	0.02
HIV RNA copies/ml	-0.35	-0.74	0.03	0.07
Executive: Adjusted R^2=0.103, Model P=0.034 [c]				
CD4 T-cells/μl	-0.001	-0.002	0.000	0.06
rs2686344	0.39	0.01	0.77	0.05
rs1718120	0.36	-0.01	0.74	0.06
Motor Speed: No individual variables achieved P<0.1				
Total Cognitive: Adjusted R^2=0.119, Model P=0.011 [c]				
Age	-0.03	-0.06	-0.01	0.02
HIV RNA copies/ml	-0.14	-0.29	0.01	0.08

Optimal models achieving an Adjusted R^2 ≥0.1 and mode P<0.05 are shown.

[a] CI: Confidence Interval;

[b] β represents the regression coefficient

[c] models retaining at least one SNP (P<0.05)

Saupplementary Table 1. The median (range) neurocognitive domain Z-scores for each genotype

	n	Cognitive Domain [a]					
		Attention	**Fluency**	**Memory**	**Executive**	**Motor Speed**	**Total**
				P2X4R			
rs2686387*G	37	-0.11 (-1.94 to 2.23)	-0.33 (-2.48 to 3.48)	-2.41 (-6.19 to 1.25)	0.93 (-2.88 to 1.57)	0.62 (-2.82 to 1.75)	-0.25 (-1.31 to 1.17)
rs2686387*C	20	-0.28 (-1.94 to 1.06)	0.38 (-2.00 to 3.24)	-2.93 (-5.40 to 0.49)	0.91 (-1.16 to 1.40)	0.96 (-2.02 to 1.43)	-0.18 (-1.57 to 0.96)
rs7298368*T	38	-0.11 (-1.94 to 1.06)	-0.22 (-2.48 to 3.48)	-2.55 (-5.71 to 1.25)	0.93 (-2.88 to 1.57)	0.59 (-2.82 to 1.64)	-0.28 (-1.31 to 1.17)
rs7298368*C	19	-0.11 (-1.94 to 2.23)	0.38 (-2.00 to 3.24)	-2.83 (-6.19 to 0.49)	0.99 (-1.16 to 1.40)	0.98 (-2.02 to 1.75)	-0.17 (-1.57 to 0.96)
				CAMKK2			
rs1653587*T	10	-0.11 (-0.53 to 1.14)	-0.45 (-2.48 to 1.57)	-3.2 (-4.38 to 0.10)	1.08 (-2.88 to 1.42)	0.64 (-2.82 to 1.50)	-0.48 (-1.08 to 0.64)
rs1653587*A	47	-0.11 (-1.94 to 2.23)	-0.10 (-2.00 to 3.48)	-2.72 (-6.19 to 1.25)	0.93 (-1.16 to 1.57)	0.76 (-2.21 to 1.75)	-0.13 (-2.05 to 1.17)
rs1653588*A	10	-0.28 (-0.78 to 0.64)	-0.57 (-2.48 to 1.33)	-3.16 (-4.38 to 0.10)	0.96 (-2.88 to 1.41)	0.68 (-2.82 to 1.50)	-0.58 (-1.08 to 0.44)
rs1653588*T	49	-0.11 (-1.94 to 2.23)	0.14 (-2.00 to 3.48)	-2.68 (-6.19 to 1.25)	0.93 (-1.16 to 1.57)	0.65 (-2.21 to 1.75)	-0.17 (-2.05 to 1.17)
rs11065504*C	27	0.23 (-1.94 to 2.23)	-0.10 (-2.00 to 3.48)	-3.02 (-6.19 to -0.13)	0.98 (-0.62 to 1.57)	0.91 (-2.02 to 1.75)	-0.19 (-2.05 to 1.15)
rs11065504*G	29	-0.11 (-1.94 to 1.14)	-0.10 (-2.48 to 3.24)	-2.13 (-5.71 to 1.25)	0.92 (-2.88 to 1.42)	0.62 (-2.82 to 1.64)	-0.25 (-1.57 to 1.17)
rs7975295*C	32	-0.11 (-1.94 to 1.14)	-0.22 (-2.48 to 3.48)	-2.07 (-5.71 to 1.25)	0.99 (-2.88 to 1.57)	0.63 (-2.82 to 1.64)	-0.20 (-1.31 to 1.17)
rs7975295*T	25	-0.11 (-1.94 to 2.23)	-0.10 (-2.00 to 3.24)	-3.25 (-6.19 to 0.49)	0.83 (-1.16 to 1.41)	0.76 (-2.02 to 1.75)	-0.36 (-2.05 to 0.81)
rs2686344*T	25	-0.11 (-1.61 to 1.14)	0.38 (-2.00 to 3.24)	-2.21 (-5.06 to 1.25)	0.99 (-0.20 to 1.42)	0.65 (-2.21 to 1.40)	-0.09 (-1.57 to 1.17)
rs2686344*C	34	-0.11 (-1.94 to 2.23)	-0.57 (-2.48 to 3.48)	-3.14 (-6.19 to 0.10)	0.93 (-2.88 to 1.57)	0.68 (-2.82 to 1.75)	-0.56 (-2.05 to 1.15)
rs1718120*G	25	-0.11 (-1.94 to 2.23)	0.38 (-2.48 to 3.48)	-3.25 (-6.19 to 1.25)	1.05 (-1.16 to 1.57)	0.94 (-1.19 to 1.75)	-0.17 (-1.34 to 1.17)
rs1718120*T	31	-0.44 (-1.94 to 1.14)	-0.33 (-2.00 to 3.24)	-2.01 (-5.71 to 0.70)	0.83 (-2.88 to 1.42)	0.54 (-2.82 to 1.50)	-0.31 (-1.57 to 0.64)

[a] Median (range)

Supplementary Table 2. Clinical features and the impact of TB on neurocognitive outcomes in Indonesians after 12 months of ART

Variable	Median (Range)
Age (years)	31 (19-48)
CD4 T-cells/μl	288 (44-763)
HIV RNA log_{10} copies/ml	1.3 (1.3-6.32)
Education (years)	12 (6-16)
History of TB	27/59 (46%)
ART[b]:	
Lamivudine	100%
Efavirenz	90%
Tenofovir	25%
Stavudine	12%
Zidovudine	23%

Domain Z-scores	**TB +ve**	**TB -ve**	**P[a]**
Median (range)	n=27/59 (46%)	n=32/59 (54%)	
Attention	-0.11 (-1.94 to 2.23)	-0.11 (-1.61 to 1.14)	0.42
Fluency	-0.81 (-1.76 to 3.48)	0.38 (-2.48 to 3.24)	0.20
Memory	-2.41 (-6.19 to 1.25)	-2.78 (-5.71 to 0.70)	0.83
Executive	0.90 (-1.16 to 1.57)	1.00 (-2.88 to 1.42)	0.36
Motor Speed	0.53 (-2.02 to 1.75)	0.76 (-2.82 to 1.50)	0.92
Total	-0.52 (-2.05 to 1.17)	-0.18 (-1.57 to 0.96)	0.46

[a] Wilcoxon rank sum test

[b] ART regimens included 3 antivirals and changes to regimens were common [1, 3]

Supplementary Table 3. SNP in *P2X4R* do not influence neurocognitive outcomes after 12 months of ART

Variable	β	95% CI[a]		P
		2.5%	97.5%	
Attention: No individual variables achieved P<0.1				
Fluency: Adjusted R^2=0.144, Model P=0.009				
Education	0.20	0.08	0.32	0.002
Memory: Adjusted R^2=0.065, Model P=0.029				
Age	-0.08	-0.15	-0.01	0.03
Log_{10} HIV RNA copies/ml	-0.35	-0.74	0.03	0.07
Executive: Adjusted R^2=0.090, Model P=0.027				
CD4 T-cells/μl	-0.001	-0.002	0.00	0.09
Education	0.08	0.01	0.14	0.02
Motor Speed: No individual variables achieved P<0.1				
Total Cognitive: Adjusted R^2=0.321, Model P=0.0002				
Age	-0.03	-0.06	-0.01	0.02
Education	0.07	0.01	0.13	0.02

[a] CI: Confidence Interval

Chapter 9

Conclusions and Future Directions

Chapter 9 outlines conclusions drawn from this thesis and considerations for future directions

9.0 Conclusions and Future Directions

9.1 Thesis Conclusions

The neurological sequelae of HIV infection remains a common burden in the lifelong management of patients. Up to 40% and 50% of individuals receiving stavudine-free ART may be affected by HIV-SN or HAND, respectively. Both complications feature increased inflammation and neuronal degeneration and so may share common mechanisms. However the precise mechanisms that lead to HIV-SN and HAND are not clear. This thesis sought demographic, clinical and genetic risk factors for developing HIV-SN or HAND, and considers how they may shed light on the pathogenesis of these conditions. To do this, I investigated the role of host genes involved in inflammatory and neuronal repair/growth pathways in Indonesians and Africans assessed for HIV-SN and/or HAND. The results have the potential to inform HIV treatment and management of those at risk, and may one day aid the rational design of therapeutic strategies. A conceptual model based on the results in thesis is presented in Figure 1.

I first assessed SNP in the *TNF*-block in Indonesians and Africans treated without stavudine. I identified that *TNF*-block genotypes associate with HIV-SN but are different to associations seen without stavudine [1-4]. This suggests differences in the underlying pathways. Furthermore, SNP associated with HIV-SN differed in Indonesians and Africans. However the haplotypes associated with HIV-SN in each cohort differed by only one allele. Haplotype analyses suggest these haplotypes may descend from a common ancestor. Therefore, it's plausible that haplotypes may share critical allele/s not typed in our panel which influences a common mechanisms contributing to HIV-SN. This study confirms a role of inflammation in the pathogenesis of HIV-SN.

I then investigated associations between the *P2X*-block genotypes and HIV-SN in Africans and Indonesians treated without stavudine. The cohorts recruited by my colleagues confirmed that the prevalence of HIV-SN in Indonesians and Africans treated without stavudine has decreased. HIV-SN in Indonesians treated without stavudine decreased by 50% (34 to 14%) and associated with markers of HIV disease severity [5]. In Africans, HIV-SN decreased from 56% to 38% and associated with higher viral loads and lower CD4 T-cell counts. Moreover, I

confirmed a role in HIV-SN for SNP in the *P2X*-block, particularly *CAMKK2*. Associated genotypes differed from those in Africans receiving stavudine [6] and between Africans and Indonesians treated without stavudine. As with *TNF*-genotypes, this may reflect the differing patterns of LD between the two populations or slight differences in the pathways leading to HIV-SN. Nonetheless, I established a clear link between HIV-SN and *CAMKK2* which warrants further investigations in larger cohorts of clearly defined ethnicities and ART regimens.

Given the strong link between *CAMKK2* and HIV-SN, we assessed associations between the *P2X*-block and large and small fibre neuropathy in a subset of the Indonesian cohort. Small fibre neuropathy associated with two SNP in *CAMKK2* whereas large fibre neuropathy associated with a single SNP in *ANAPC5*. This suggests a link between *CAMKK2* and small fibre pathology in HIV-SN and warrants further assessment.

In light of associations between the *P2X*-block and HIV-SN, I compared the expression patterns of P2X7R, P2X4R and CaMKK2 and the IENFD in skin samples from Indonesian patients with and without HIV-SN, and healthy controls. IENFD were significantly lower in samples from HIV+ individuals with and without HIV-SN compared to healthy controls. This is consistent with neurotoxicity associated with HIV infection. P2X7R, P2X4R and CaMKK2 expression differed for each protein and varied by HIV-SN status. An important finding is that CaMKK2+ cells were more common in patients with HIV-SN than those without, and most CaMKK2+ cells were co-located with nerves. The differences in expression and the close vicinity to nerves supports a neurological role for CaMKK2 in HIV-SN, and further substantiates the genetic evidence linking the *P2X*-block to HIV-SN.

I next investigated whether *P2X*-block SNP associated with HIV-SN in Indonesians and Africans were also markers of risk for HAND in HIV+ Indonesians as they commenced ART and in the first 12 months of treatment. Interestingly, no *P2X4R* SNP associated with HAND. However, only two SNP from *P2X4R* were included in the final analyses and effects of *P2X4R* may be mediated by SNP in LD in *CAMKK2* and *P2X7R*. Two intronic SNP in *CAMKK2* were associated with altered domain-specific neurocognitive outcomes after 12 months on ART. One SNP, which associated with higher executive function scores in Indonesians, also associated with higher speed information processing scores in HIV+ Australians, supporting a link between

CAMKK2 and neurocognitive impairment. Five intronic *P2X7R* SNP associated with neurocognitive outcomes in a time- and domain-specific manner. Based on existing literature, each of the five SNP may plausibly contribute to neurocognitive outcomes. These results were again partially replicated in Australian HIV+ patients, highlighting *P2X7R* as a promising candidate for future investigations into the pathogenesis of HAND. Validation of these findings in larger independent cohorts and multiple ethnicities is warranted.

9.2 Future Directions

This thesis builds on the work of my colleagues and of other groups, providing further evidence that susceptibility to HIV-SN is influenced by the *TNF*-block genotype and highlights a link with the *TNF*-block that is independent of stavudine use. Furthermore, this thesis provides strong evidence for a role of the *P2X*-block in HIV-SN and HAND. A clear relationship is established with *CAMKK2* in HIV-SN and an early link with *P2X7R* in HAND. Furthermore, this thesis presents preliminary evidence that associations with P2X-block genotypes may be mediated in HIV-SN by the encoded proteins.

Future investigations should aim to identify critical *P2X*-block genotypes and confirm a role for the encoded proteins in the inflammatory and neurotoxic pathways underpinning both HIV-SN and HAND. Further genetic investigations of HIV-SN and HAND utilising a more comprehensive gene and SNP panel (including the *TNF*-block), genome-wide association studies or deep sequencing of large, longitudinal cohorts and in multiple ethnicities are warranted. In silico modelling of key SNP associated with HIV-SN and HAND could identify possible pathogenic variants which could be validated utilising expression constructs.

Assessment of the expression patterns of P2X7R, P2X4R and CaMKK2 in biopsies from the lower leg of patients with and without HIV-SN provides invaluable insights of the physical relationship between nerves and proteins with minimal impact to the patient. Validation of expression patterns of P2X7R, P2X4R and CaMKK2 with additional markers of immune cells and epithelium to clearly identify expressing cells is required. Furthermore, future studies should address longitudinal cohorts of known *P2X*-block genotype as expression of the proteins may vary during the development of HIV-SN. This should be linked with clinical

features such as numbness and pain. Quantitation of protein and mRNA expression from biopsy material will provide additional information.

Understanding the role of the encoded proteins and the expression patterns of the *P2X*-block in the brain of individuals with HAND would also be of great value. However, post-mortem tissue is very limited and provides only a snapshot into the pathology of the later stages of HAND. Assessment of proteins in blood or cerebrospinal fluid may not capture time- or tissue-specific expression of the encoded proteins. Therefore, further genetic investigations, as discussed above, in silico modelling, and studies utilising animal models are required to further the understanding of the pathogenic mechanisms of HAND and identify potential therapeutic targets.

Finally, this study highlights potential common pathways between two HIV-associated neurological conditions. Associations with the *P2X*-block and the peripheral and central nervous systems may be independent of HIV and so the *P2X*-block may influence other neurodegenerative diseases. Indeed the literature supports a links with Alzheimer's disease, schizophrenia and multiple sclerosis [7-10]. We recently reported that expression of P2X7R, P2X4R and CaMKK2 in neurons in the frontal cortex of post-mortem tissue did not differ between donors with and without Alzheimer's disease (Appendix 1; [11]) Expression may vary within specific regions of AD brain or be restricted to earlier stages of AD pathology. Histological investigation is limited by the availability of samples from younger donors. Therefore, we are now exploring the genetic signature of the *P2X*-block in the progression of Alzheimer's disease.

Figure 1.

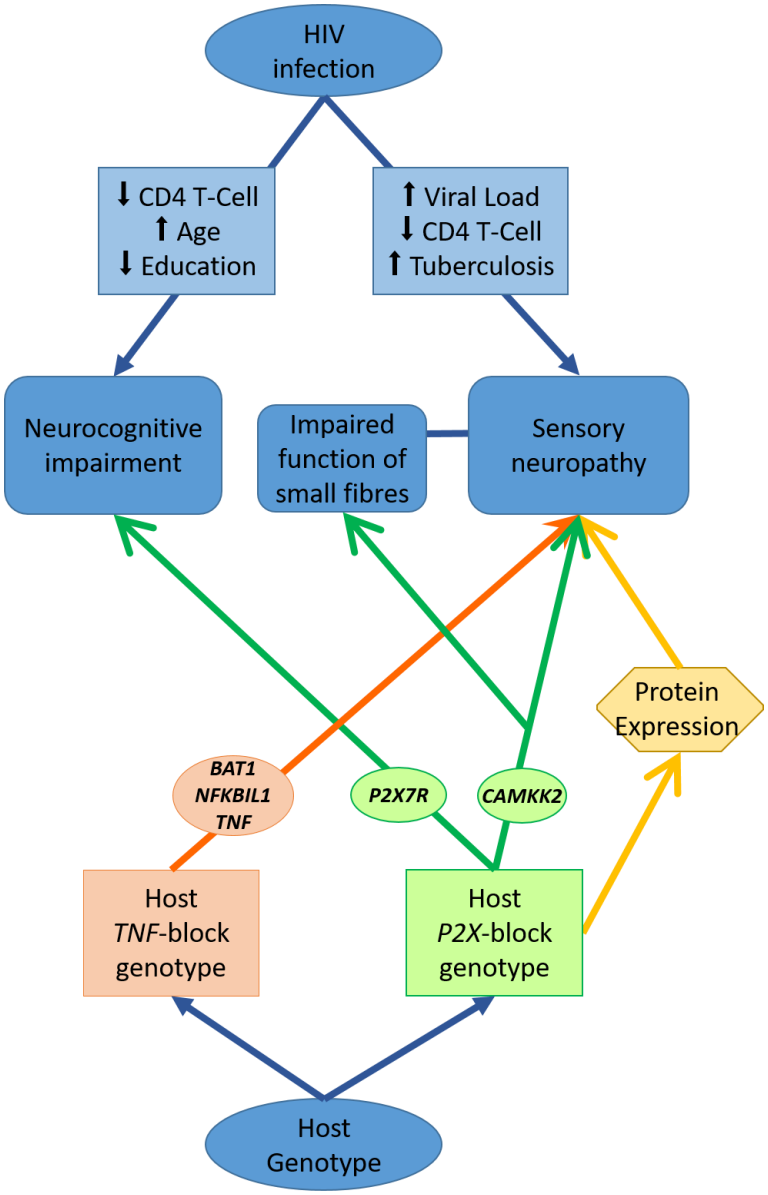

Figure 1. Conceptual model based on the results in thesis

This thesis identified demographic, clinical and genetic markers of risk for HIV-SN and HAND in Indonesians and South Africans. SNP in *TNF*, *NFKBIL1*, and *BAT* within the *TNF*-block associated with HIV-SN. A clear association was identified between *CAMKK2* in the *P2X*-block and HIV-SN, notably with impairment of small fibres and SNP within *P2X7R* associated with HAND. However, associating SNP differed between Indonesians and South Africans and further investigation is required to identify critical genotypes.

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Appendix 1

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Short report

Immunohistochemical evidence of P2X7R, P2X4R and CaMKK2 in pyramidal neurons of frontal cortex does not align with Alzheimer's disease

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ABSTRACT

Alzheimer's disease (AD) is an incurable neurodegenerative condition resulting in progressive cognitive decline. Pathological features include Aβ plaques, neurofibrillary tangles, neuroinflammation and neuronal death. Purinergic receptors 7 and 4 (P2X7R and P2X4R) and calcium/calmodulin-dependent kinase kinase 2 (CaMKK2) are implicated in neuronal death. We used immunohistochemistry to investigate the distribution of these proteins in neurones from frontal cortex of donors (n = 3/group; aged 79–83 years) who died with and without AD. Neurones were identified morphologically and immunoperoxidase staining was achieved using commercial antibodies. Immunoreactive neurones were counted for each protein by 2–3 raters blinded to the diagnoses. We observed no differences in percentages of P2X7R, P2X4R or CaMKK2 positive neurones (p = 0.2–0.99), but sections from individuals with AD had marginally fewer neurones (p = 0.10). Hence P2X7R, P2X4R or CaMKK2 appear to be expressed in neurones from older donors, but expression does not associate with AD.

1. Introduction

Alzheimer's disease (AD) is an incurable neurodegenerative disease resulting in a progressive cognitive decline which impairs basic functional abilities (Mattson, 2004). Neuropathological hallmarks of AD include extracellular deposition of the neurotoxic beta amyloid peptide 1-42 (Aβ) in the form of senile plaques, and the appearance of intracellular neurofibrillary tangles composed of the hyperphosphorylated microtubule-associated protein tau (Selkoe and Hardy, 2016). AD is further characterised by dysregulated neuroinflammation, calcium dyshomeostasis, increased oxidative stress, and synaptic and neuronal dysfunction triggered by cell death (Akiyama et al., 2000; Huang et al., 2016; Kawahara et al., 2009). Underlying mechanisms remain unclear. Purinergic receptors 7 and 4 (P2X7R and P2X4R) and calcium/calmodulin-dependent kinase kinase 2 (CaMKK2) are implicated in neuronal death and warrant investigation in brains affected by AD.

Several recent reviews describe a role in AD for purinergic receptors, including P2X7R and P2X4R (Godoy et al., 2019; Cieślak and Wojtczak, 2018; Francistiová et al., 2020). P2X7R and P2X4R are ATP-gated non-specific cation channels activated by Aβ-induced release of extracellular ATP triggering influx of calcium ions (Ca^{2+}) (Parvathenani et al., 2003; Varma et al., 2009). In mice, microglial activation of P2X7R and Ca^{2+} influx induce synthesis and release of proinflammatory cytokines including TNFα (Shieh et al., 2014) which is found at elevated levels in AD (Akiyama et al., 2000). In co-cultures and rodent models of AD, P2X7R-dependent activation of microglia and influx of Ca^{2+} increase levels of reactive oxygen species and neuronal death (Parvathenani et al., 2003). Inhibition of P2X7R in rodent models of AD reduced Aβ plaques (Diaz-Hernandez et al., 2012) and neuronal death (Ryu and McLarnon, 2008).

The role of P2X4R in AD is less clear. In neuroinflammatory settings, P2X4R is upregulated in spinal and brain microglia in patients with multiple sclerosis – this was replicated in microglial cultures stimulated in vitro (Vázquez-Villoldo et al., 2014). Moreover the downregulation of spinal expression of P2X4R in mice diminished production of proinflammatory cytokines (Xu et al., 2018). P2X4R may also play a role in

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neurotoxicity attributed to sustained Ca^{2+} influx in AD. Cultured hippocampal neurones treated with neurotoxic Aβ trigger upregulation of full length and c-terminal cleaved P2X4R in neuronal cell bodies and neurites. Cleavage of P2X4R delays channel closure, permitting greater influx of Ca^{2+} and subsequent neuronal death. Moreover, cultures overexpressing P2X4R exhibited greater cytosolic Ca^{2+} levels and Aβ-induced neurotoxicity than cultures with reduced P2X4R expression (Varma et al., 2009).

CaMKK2 is a serine/threonine kinase expressed abundantly in the brain. CaMKK2 phosphorylates and activates AMP-activated kinase (AMPK), which phosphorylates tau at S262. Phosphorylation at S262 induces dendritic spine loss in cultured hippocampal neurones exposed to Aβ, whereas inhibition of CaMKK2 and/or AMPK prevented S262 phosphorylation and Aβ-associated neurotoxicity (Mairet-Coello et al., 2013). Furthermore, CaMKK2-activated AMPK is found abundantly in dystrophic neurites surrounding Aβ plaques and in tangle- and pre-tangle- bearing neurones in patients with AD, suggesting involvement in AD progression (Vingtdeux et al., 2011).

Genetic evidence further supports a role for the three proteins. A single nucleotide polymorphism (SNP) within the gene encoding P2X7R associated with reduced risk of AD (Sanz et al., 2014). Furthermore, a SNP in the gene encoding CaMKK2 associated with lower expression of CaMKK2 in dorsolateral prefrontal cortex, increased risk of schizophrenia, and deficits in cognitive function in schizophrenics (Yu et al., 2016). We have associated SNP in all three genes with altered risk of HIV-associated sensory neuropathy, a neurodegenerative condition affecting peripheral nerves (Gaff, 2019; Gaff et al., 2020).

Although P2X7R, P2X4R and CaMKK2 are linked with neuronal pathology in animal models of AD and in models based on cultured neurones, the expression of these proteins in human brain affected by AD is uncertain. Using immunohistochemistry, we describe the distribution of the three proteins in neurones from frontal cortex of donors who died with or without AD.

2. Materials and methods

2.1. Ethics and sample information

This research and the use of human tissue was approved by the Human Research Ethics Office of Curtin University (HRE2018-0318). Post-mortem central nervous system tissues, specifically, formalin fixed paraffin embedded frontal cortex from Brodmann areas 11 and 12 were provided for three donors diagnosed with clinical AD (Braak stage V, V and VI) and three age and gender-matched non-AD donors (Braak stage 0, 0 and I) by the Victorian Brain Bank (VBB) at the Florey Institute for Neuroscience and Mental Health.

2.2. Sample preparation

Three serial sections (~10mm^2, 5 μm thick) from each of the donors were deparaffinised (5 min, 3 changes xylene; 534,056; Sigma, Missouri, USA), rehydrated (2 × 10 min in 100, 95, 70, and 50% ethanol; ET00052500; Scharlab, Barcelona, Spain) and washed in MilliQ water (2 × 5 min). For antigen retrieval, sections were submerged in 10 mM sodium citrate buffer (trisodium citrate dehydrate; pH = 6.0; S1804; Sigma) with 0.05% Tween 20 (P1379; Sigma), microwaved for 10 min and washed in MilliQ water (2 × 5 min). Endogenous peroxidases were blocked using 1% hydrogen peroxide (H1009; Sigma) at room temperature for 10 min, followed by 5 min in phosphate-buffered saline (PBS; P4417; Sigma). Sections were incubated for 10 min in avidin/biotin blocking reagents (004303; Life Technologies, California, USA) and washed for 5 min in PBS. Samples were incubated overnight in 5% normal donkey serum (NDS) in PBS at 4 °C to reduce non-specific binding of antibodies.

2.3. Immunohistochemistry

Three serial sections were treated with antibodies; goat anti-P2X7R at 5μg/ml (ab105047; Abcam, Cambridge, UK), anti-P2X4R at 5μg/ml (ab134559; Abcam) or anti-CaMKK2 at 2 μg/ml (sc-9629; Santa Cruz Biotechnology, Texas, USA) diluted in PBS with 1% NDS (2 h at room temperature). The specificity of the antibodies was validated by Immunohistochemistry and Western Blot in studies cited by the manufacturer (Asif et al., 2019; Briski et al., 2017; Chessell et al., 1998). Sections were then washed in PBS (3 × 5 min) before incubation with donkey anti-goat IgG conjugated with biotin (ab6884; Abcam) diluted to 20 μg/ml in PBS with 1% NDS for 1 h at room temperature, followed by PBS washes (3 × 5 min). This was visualised using streptavidin labelled with horseradish peroxidase (BD Pharminogen, California, USA) diluted 1:200 in PBS plus 1% NDS (30 min at room temperature). Sections were washed in PBS (3 × 5 min) and treated with 3, 3′ diaminobenzidine (DAB; D4293, Sigma) dissolved in MilliQ water and applied for 12 min (following anti-CaMKK2) or 8 min (following anti-P2X7R or -P2X4R). After washing in PBS and MilliQ water sections were counterstained with Gill's Haematoxylin (30 s), washed in running tap water, dehydrated for 1 min in 70%, 95% and three changes of 100% ethanol, and cleared for 1 min in three changes of xylene. Sections were mounted with Entellan New (Proscitech, Queensland, Australia) and glass coverslips (#1.5; Proscitech). Samples treated without primary antibodies were included as negative controls.

2.4. Imaging and analyses

Brightfield images were collected using an Olympus UPlanSApo 40× NA0.75 objective on an Olympus BX-51 (Olympus Corporation, Tokyo, Japan) equipped with a DP70 camera (Olympus) and Olympus cellSens Standard software version 3.14 for Windows (Fig. 1). Whole-section digital images were obtained using a Leica (Aperio) Scanscope XT® slide Scanner (Aperio Technologies, California, USA) with an Olympus UPlanSApo 20× NA0.75 objective. From whole slide images, three to five fixed size (110,000μm^2), random fields containing layer V pyramidal neurones were extracted as individual images using the Aperio ImageScope software (Version 12.3.2.8013 for Windows; Aperio Technologies Inc). Immunoreactive counterstained cells with the morphological features of layer V pyramidal neurones were defined as 'positive' and non-immunoreactive cells as 'negative'. All positive and negative neurones in every image were counted by 2–3 raters blinded to AD diagnoses. Percentages of positive neurones were assessed using Mann-Whitney tests in GraphPad Prism version 8.2.1 for Windows (Graphpad Software, California, USA). Intraclass correlation coefficients were calculated (two-way random, average measures and absolute agreement) using the "irr" package (Gamer et al., 2019).

3. Results and discussion

Donors with and without AD were matched by age (79.9–82.9 vs 79.0–82.7 years, resp.) and proportion of males (33% vs 33%, resp.; Table 1). Brightfield images (40× objective) were used to define neurones as positive or negative based on morphological features (distinct pyramidal soma and visible nucleus and nucleolus) and immunoreactivity (Fig. 1). Cells which could not be identified morphologically were excluded from our counts. Comparisons with sections stained without primary antibodies established that staining detected with all three antibodies reflected expression of P2X4R, P2X7R and CaMKK2 in all sections from donors dying with and without AD (Fig. 1). Sections from one donor with AD exhibited high background staining with P2X4R and were excluded.

The average percentage of positive neurones per sample was determined for each rater (JG, PP, SW) and intraclass correlation coefficients were calculated for each protein. Intraclass correlation coefficients of 0.60, 0.72 and 0.83 for CaMKK2, P2X7R and P2X4R (resp.) indicate

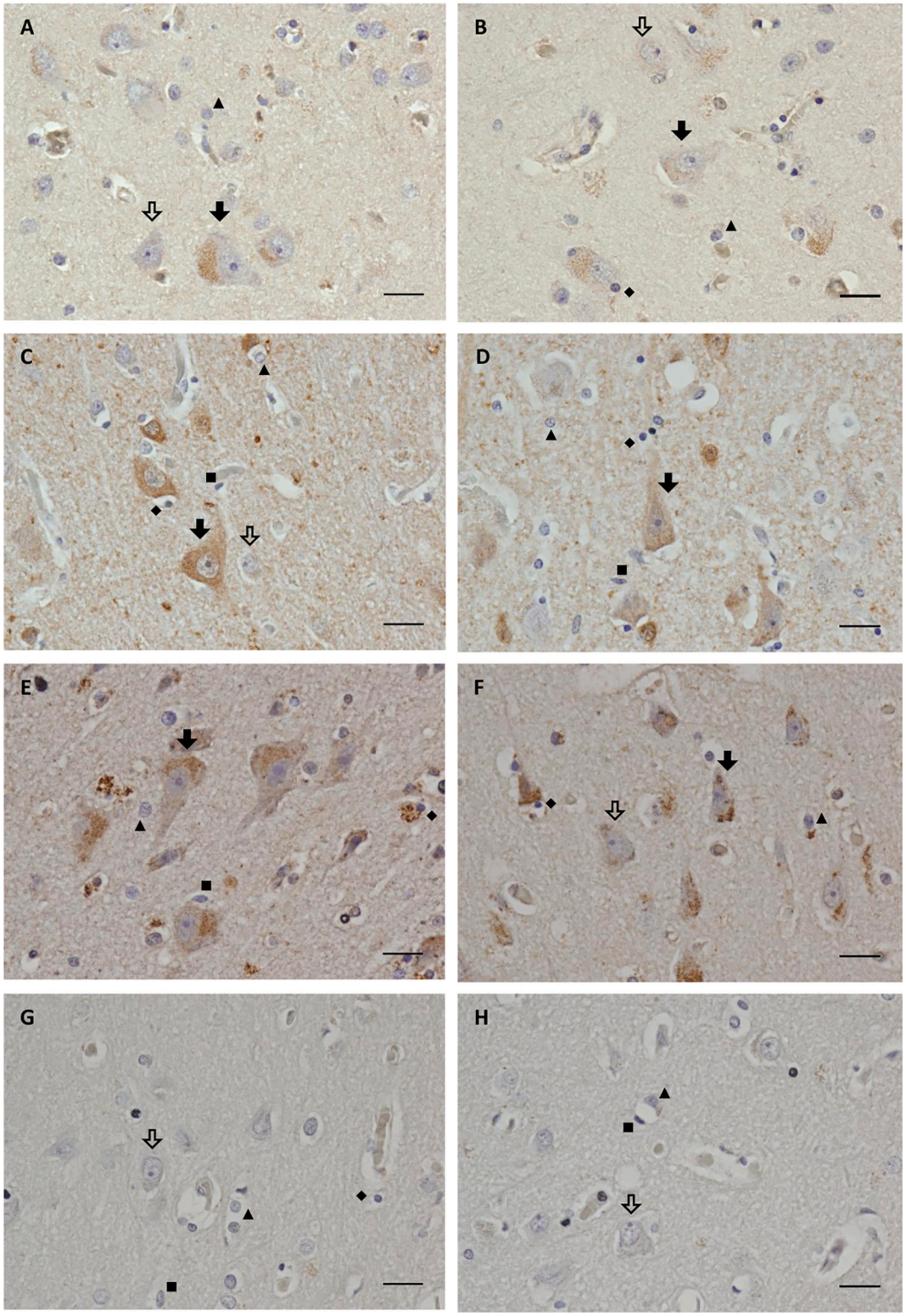

Fig. 1. A–H. Immunohistochemical staining of frontal cortex from AD (A, C, E, G) and non-AD (B, D, F, H) donors. Cells defined as positive and negative neurones are indicated with filled and open arrows, respectively. Cells with the morphological features of astrocytes (triangles), oligodendrocytes (diamonds) and microglia (squares) were observed adjacent to neurones. Neurones from AD and Non-AD donors were immunoreactive for P2X7R (A–B), P2X4R (C–D), and CaMKK2 (E–F). Minimal immunoreactivity was observed in negative control tissue treated without primary antibodies (G–H). Scale bars indicate 20 μm. Abbreviations: *AD;* Alzheimer's disease, *P2X7R;* purinergic receptor 7, *P2X4R;* purinergic receptor 4, *CaMKK2;* calcium/calmodulin dependent kinase kinase 2.

Table 1
Proportions of immunoreactive neurones were unaffected by AD.

				Percentage Positive Neurones			Total neurones counted[a,b]
Donor	Braak Stage	Gender	Age (years)	P2X7R[a]	P2X4R[a]	CaMKK2[a]	
AD	V	Female	79.9	74.7 (72.1-76.6)	-[d]	80.7 (69.2-88.1)	12.7 (11.9-13.5)
AD	V	Female	82.9	80.5 (79.3-81.0)	93.0 (91.7-94.2)	88.0 (79.6-89.7)	19.5 (16.5-23.5)
AD	VI	Male	82.3	75.1 (70.7-80.6)	86.1 (84.3-87.8)	95.1 (81.0-97.9)	12.2 (8.8-15.4)
Non-AD	0	Female	79.0	84.9 (83.7-88.5)	81.6 (81.3-81.8)	84.3 (72.1-85.3)	23.8 (18.7-27.2)
Non-AD	I	Female	81.2	87.8 (75.1-88.0)	83.9 (81.8-86.1)	91.9 (87.0-92.7)	19.7 (18.4-21.3)
Non-AD	I	Male	82.7	73.7 (50.8-74.1)	57.7 (50.7-64.8)	91.2 (87.0-92.7)	22.2 (16.5-30.7)
			p=0.70[c]	*p=0.70*[c]	*p=0.20*[c]	*p=0.99*[c]	*p=0.10*[c]

[a] Results are presented as median (range).
[b] Total neurones per field counted for each donor.
[c] Mann-Whitney test AD versus Non-AD ($n = 9$ reflecting replicate sections).
[d] This sample had high backgrounds so additional fields were counted from the other donors.

satisfactory correlation between counts (data not shown). Most neurones were positive for P2X7R, P2X4R and CaMKK2 (57–95% positive; Table 1), so many neurones must express 2–3 of the proteins. Indeed, coexpression of P2X7R and P2X4R is well documented (Koo and Li, 2016). Proportions of positive neurones calculated for each protein show no significant differences between AD and non-AD tissue (p = 0.20–0.99; Table 1). Sections from individuals with AD had slightly fewer neurones than controls, but the difference was not significant (p = 0.10).

While AD did not affect P2X7R, P2X4R and CaMKK2 immunoreactivity in neurones within the frontal cortex, expression may vary between regions of the brain or be restricted to earlier stages of AD pathology. In a prior study (Varma et al., 2009), P2X4R levels were decreased in the medial frontal gyrus and the medial temporal gyrus of donors with severe AD pathology compared with non-AD donors, but no difference was observed in the cerebellum. Moreover, P2X4R expression was upregulated following exposure to Aβ but prior to neuronal cell death in an in vitro model of AD. In a familial AD mouse model, transcription of P2X7R in neurones of the dentate gyrus was reduced in the early and advanced AD, but normalised in late stage AD (Martínez-Frailes et al., 2019). Patients in this study, aged 79.9–82.9, had a diagnosis of AD for around 7 years, but neurological changes may have been initiated before that time. Donors without a diagnosis of AD were similar in age (aged 79.0–82.7 years) and may display age-related changes.

Cells with the morphological features of astrocytes, oligodendrocytes and microglia were often observed adjacent to neurones, but immunoreactivity was rarely clear in glial cells (Fig. 1). Expression of P2X7R, P2X4R and CaMKK2 is reported in astrocytes and microglia in rodent brain and in human cultures (Zhang et al., 2018; Burnstock, 2008). Dual labelling with cell-specific markers is warranted to assess these lineages in human brain.

Although our study was small, we observed P2X7R, P2X4R or CaMKK2 immunoreactivity in frontal cortex pyramidal neurones from all six donors. No differences between individuals with and without AD were apparent and most neurones in all sections expressed the three proteins. Further investigations will be limited by the availability of clinical material collected from younger donors, so it is appropriate to view our data in the context of studies based on animal models. In an alternative approach, we are now examining associations between AD and polymorphisms in the genes encoding P2X7R, P2X4R or CaMKK2.

Author statement

JG optimized the techniques, performed the immunohistochemistry and created the images, CJ assisted with optimization of the histochemistry, JP viewed the images as a pathologist, SW counted neurones, CM provided the histological sections and associated clinical details, PP coordinated the project.

Declaration of Competing Interest

None.

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Appendix 2

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Polymorphisms in *P2X4R* and *CAMKK2* may affect TNFα production: Implications for a role in HIV-associated sensory neuropathy

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ABSTRACT

Polymorphisms in *P2X4R* and *CAMKK2* associate with susceptibility to HIV-associated sensory neuropathy (HIV-SN) – a condition likely mediated by TNFα. As single nucleotide polymorphisms (SNPs) and haplotypes of *CAMKK2*, and a neighbouring gene *P2X4R*, mark susceptibility to HIV-SN in South Africans living with HIV, we examined the relationship between *P2X4R* and *CAMKK2* genotypes and TNFα production. Peripheral blood mononuclear cells from 129 healthy donors were stimulated with killed *Escherichia coli,* and concentrations of soluble TNFα were assessed. Their DNA was genotyped for 22 SNPs in *P2X4R* and *CAMKK2*. Three SNPs within *P2X4R* and two SNPs within *CAMKK2* influenced concentrations of TNFα, but these SNP did not associate with risk for HIV-SN. This incongruence may reflect differences in *P2X4R* haplotypes present in Africans and Europeans. However some *CAMKK2* haplotypes were found in both populations, so *CAMKK2* polymorphisms may impact upon HIV-SN via effects of the protein on pathways other than TNFα.

1. Introduction

HIV-associated sensory neuropathy (HIV-SN) is a neurological complication occurring in up to 60% of HIV + individuals. It is a length-dependant disease predominately affecting the nerve fibres that innervate the distal limbs, particularly the feet [1]. Symptoms include pain, burning, and numbness, which impact an individual's quality of life and work capabilities [2,3]. Susceptibility has been linked to single nucleotide polymorphisms (SNPs) and haplotypes in the *P2X4R* and *CAMKK2* genes in patients of African descent [4]. However genetic analyses, post-mortem studies in humans, and animal models implicate tumour necrosis factor alpha (TNFα) in the underlying pathology [5–8]. We seek a link between TNFα, *P2X4R* and *CAMKK2*.

CAMKK2 and *P2X4R* are contiguous genes located in a region of high linkage disequilibrium on chromosome 12. *CAMKK2* encodes calcium/calmodulin-dependant protein kinase kinase 2 (CaMKK2), a protein with roles in cellular metabolism, neuronal repair and inflammation [9,10]. Little is known about the expression of CaMKK2 in immune cells, but it appears, at least in mice, to be limited to monocytes and macrophages. CaMKK2 activation by intracellular Ca^{2+} propagates CaM kinase signalling cascades, including CaM kinase IV (CaMKIV)-induced TNFα production, via p38-MAPK and Activation Factor 1. Indeed, ablation of *CAMKK2* in macrophages from knockout mice diminished toll-like receptor 4 (TLR4) signalling after stimulation with lipopolysaccharide (LPS), impairing synthesis of inflammatory chemokines and cytokines including TNFα [9].

P2X4R encodes a purinergic receptor 4 (P2X4R) which is a ligand-gated ion channel implicated in inflammatory signalling and synaptic transmission in the central nervous system. P2X4R is abundant in macrophages and microglia, where tissue insults trigger release of extracellular adenosine triphosphate (ATP) and activate P2X4R at the cell surface, initiating ion permeability. Activation of P2X4R triggers TNFα production via the p38-MAPK cascade or Brain Derived Neurotrophic Factor (BDNF) [11]. P2X4R expression was upregulated in microglial cultures after stimulation with LPS, placing the gene in a TLR4-TNFα pathway [12]. It is plausible that SNP affecting a TLR4-TNFα pathway may affect HIV-SN as TLR4-null mice exhibited attenuation of neuropathic-like hypernociception [13]. This is tested here using cells stimulated with *E. coli.*

We address the possibility that the observed associations between polymorphisms in *CAMKK2* and *P2X4R* and HIV-SN [4] may be mediated through TNFα. Overall CAMKK2 exhibited the strongest

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Table 1
Three SNPs in *P2X4R* and 2 in *CAMKK2* affect TNFα levels in cultures stimulated with killed *E. coli*.

SNP ID[a]	HIV-SN[b]	1,1	1,2	2,2	P-Value[c]		
					1,1 vs 1,2	1,1 vs 2,2	1,2 vs 2,2
P2X4R							
rs2686387[d] C/G (0.30)[e]	P > 0.20 RR = 0.70	0.95[f] (0.01–5.37) n = 61	1.85 (0.03–4.90) n = 54	1.30 (0.41–4.00) n = 10	0.006	0.30	0.30
rs2303998 G/A (0.03)	–[g]	1.27 (0.59–4.00) n = 118	1.55 (0.01–5.37) n = 8		0.38[h]		
rs7298368 C/T (0.18)	P > 0.20 RR = 0.51	1.25 (0.01–5.37) n = 86	1.68 (0.03–4.90) n = 33	0.74 (0.03–1.19) n = 6	0.07	0.16	0.024
rs25643 T/C (0.40)	P > 0.20 RR = 0.89	0.98 (0.01–5.37) n = 43	1.52 (0.03–4.90) n = 64	1.50 (0.03–4.00) n = 1 9	0.29	0.25	0.80
rs10849860 T/C (0.13)	P > 0.20 RR = 0.92	0.97 (0.01–5.37) n = 94	1.70 (0.05–3.99) n = 33		0.034		
rs11608486 T/C (0.14)	P > 0.20 RR = 0.89	0.90 (0.00–5.37) n = 86	1.05 (0.00–4.00) n = 32		0.92		
rs7961979 C/A (0.11)	P > 0.20 RR = 1.11	1.04 (0.00–5.37) n = 93	0.99 (0.00–3.26) n = 26		0.74		
CAMKK2							
rs1653587 A/G (0.07)	P = 0.12 RR = 1.80	1.29 (0.01–5.37) n = 107	1.30 (0.03–4.00) n = 18		0.52		
rs1653588 T/A (0.06)	P > 0.20 RR = 1.61	1.27 (0.01–5.37) n = 112	1.68 (0.03–3.17) n = 15		0.58		
rs11065502 G/C (0.16)	P > 0.20 RR = 1.01	0.95 (0.00–5.37) n = 86	1.10 (0.00–4.00) n = 38		0.97		
rs11065504[d] C/G (0.35)	P > 0.20 RR = 0.00	1.38 (0.03–5.37) n = 53	1.49 (0.01–4.90) n = 52	0.58 (0.01–4.72) n = 17	0.65	0.04	0.15
rs7975295 T/C (0.12)	P = 0.007 RR = 0.68	1.31 (0.01–5.37) n = 93	1.43 (0.03–4.90) n = 25		0.75		
rs2686344 C/T (0.28)	P = 0.018 RR = 1.67	1.21 (0.03–4.72) n = 67	1.27 (0.01–3.86) n = 50	1.30 (0.05–5.37) n = 11	0.36	0.98	0.65
rs1560568 G/A (0.11)	P = 0.023 RR = 0.71	1.29 (0.01–5.37) n = 103	1.43 (0.03–4.90) n = 25		0.64		
rs7314454 C/T (0.12)	P > 0.20 RR = 0.56	0.98 (0.01–5.37) n = 90	1.76 (0.03–4.00) n = 27		0.017		
rs1718120[d] G/T (0.50)	P > 0.20 RR = 0.00	1.05 (0.00–4.00) n = 26	1.02 (0.00–4.72) n = 60	0.60 (0.00–5.37) n = 25	0.57	0.40	0.88
rs3817190[d] A/T (0.37)	P = 0.19 RR = 1.91	1.29 (0.01–5.37) n = 39	1.45 (0.03–4.72) n = 44	1.36 (0.15–4.00) n = 14	0.76	0.49	0.33

[a] In chromosomal order.
[b] Association with carriage of the minor allele and HIV-SN in South Africans [4] P values (chi^2 tests) and Relative Risk (RR) are shown.
[c] Mann Whitney statistics comparing concentrations of TNFα between genotypes.
[d] The major and minor alleles of these SNP were reversed in the African population relative to Australian Caucasians. The minor allele of rs2686387 in P2X4R associated weakly with increased risk of HIV-SN in univariate analyses (p = 0.15) [4]. Rs11065504, rs1718120 and rs3817190 did not affect HIV-SN in African patients.
[e] Major/minor allele (minor allele frequency) from samples successfully genotyped.
[f] Median (range) concentrations of TNFα (ng/ml) in culture supernatants at 4 h.
[g] Allele 2 was not found in patients with HIV-SN.
[h] (1,2) and (2,2) were merged when < 5 individuals carried the (2,2) genotype.

associations with HIV-SN, with two SNPs and six haplotypes predicting HIV-SN status in South Africans of African descent. Using immunohistochemistry, we confirmed that cultured CD14+ macrophages isolated from human PBMC express P2X4R and CaMKK2 (Supplement 1). Here we explore whether genotypes associated with HIV-SN align with variations in TNFα production *in vitro*.

Table 2
One *P2X4R* and *CAMKK2* haplotype affected TNFα levels in cultures stimulated with killed *E. coli*.

Haplotype Sequence[a]	South African[b]	Australian Caucasian[c]	TNFαlevels[d]		P-value[e]
			Haplotype Absent	Haplotype Present	
		P2X4R			
111111	0%	55%	1.10 (0.03–4.00) n = 101	1.39 (0.01–5.37) n = 28	0.47
2122111	0%	15%	1.26 (0.01–5.37) n = 93	1.51 (0.03–4.90) n = 36	0.24
1112122	0%	13%	1.29 (0.01–5.37) n = 104	1.23 (0.03–3.26) n = 25	0.68
2112211	0%	12%	0.98 (0.01–5.37) n = 106	2.08 (0.06–3.00) n = 23	0.01[f]
2212221	0%	2%	1.28 (0.01–5.37) n = 123	1.55 (0.59–4.00) n = 6	0.56
		CAMKK2			
1111121111	0%	16%	1.14 (0.01–4.90) n = 100	1.50 (0.03–5.37) n = 29	0.33
1112111111	0%	12%	1.29 (0.01–5.37) n = 92	0.95 (0.01–4.90) n = 37	0.56
1112111122	18%	12%	1.29 (0.01–5.37) n = 99	1.39 (0.03–4.72) n = 30	0.52
1121111122	0%	11%	1.29 (0.01–5.37) n = 108	1.23 (0.03–3.18) n = 21	0.77
1111212111	0%	7%	1.28 (0.01–5.37) n = 113	1.62 (0.09–4.90) n = 16	0.56
1111111111	0%	7%	1.23 (0.01–5.37) n = 119	2.08 (0.05–2.84) n = 10	0.32
2211111121	3%	4%	1.29 (0.01–5.37) n = 121	1.23 (0.03–2.71) n = 8	0.84
1111111222	1%	3%	1.26 (0.01–5.37) n = 125	1.89 (1.68–2.49) n = 4	0.24
1111121121	1%	2%	1.28 (0.01–5.37) n = 125	1.68 (0.60–3.00) n = 4	0.47
1111111122	0%	2%	1.28 (0.01–5.37) n = 125	2.37 (0.15–3.26) n = 4	0.32
1112121111	0%	2%	1.30 (0.01–5.37) n = 127	0.35 (0.28–0.42) n = 2	0.18
1111121222	2%	2%	1.26 (0.01–5.37) n = 126	1.67 (0.05–3.86) n = 6	0.40
1112121122	4%	2%	1.33 (0.01–5.37) n = 123	0.03 (0.01–0.55) n = 6	0.0003[g]
1112111121	7%	2%	1.33 (0.01–5.37) n = 125	0.49 (0.07–1.18) n = 4	0.13
2211111111	0%	1%	1.29 (0.01–5.37) n = 124	1.50 (0.59–3.03) n = 5	0.46
1111212121	3%	1%	1.29 (0.01–5.37) n = 126	0.90 (0.03–1.68) n = 3	0.36
1121111121	0%	1%	1.30 (0.01–5.37) n = 127	0.94 (0.62–1.26) n = 2	0.64
1121111222	0%	1%	1.27 (0.01–5.37) n = 124	2.76 (0.74–4.00) n = 5	0.08

[a] Defined by alleles of SNPs in the order shown in Table 1.
[b] The fastPHASE haplotype frequency in the South African HIV+ population [4].
[c] The fastPHASE haplotype frequency in the Australian Caucasian population.
[d] Median (range) concentrations of TNFα (ng/ml) in cultures stimulated with killed *E. coli* at 4 h.
[e] Mann Whitney statistics comparing concentrations of TNFα between genotypes.
[f] Includes minor alleles of rs2686387 and rs10849860 associated with increased TNFα.
[g] Includes the minor allele of rs11065504 and major allele of rs7314454 associated with reduced TNFα.

2. Materials and methods

Healthy adult donors (n = 129) declaring European descent were recruited in Western Australia with approval from the Royal Perth Hospital Human Research Ethics Committee. Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll Hypaque gradients and cryopreserved. 5×10^5 PBMC were stimulated with $1x10^7$ cfu/ml heat-killed *E. coli* and incubated at 37 °C in 5% CO2 for 4, 8 or 24 h, in parallel with unstimulated PBMC. Soluble TNFα concentrations in the supernatants were measured using Duoset ELISA Development System (R&D Systems, Minneapolis, MN) [14]. DNA was extracted using QIAmp DNA mini Blood Kits (QIAGEN, Valencia, CA) and typed using OpenArray SNP kits (Thermo Fisher Scientific, Waltham, MA), designed to assess SNPs in *P2X4R* and *CAMKK2* [4]. The major and minor alleles in the European population are denoted 1 and 2 (respectively). SNPs outside Hardy-Weinberg Equilibrium or with no call outputs were excluded from analysis. Haplotypes with a frequency greater than 1% were derived from the genotypes using fastPHASE [15].

3. Results

Median (range) TNFα concentrations in unstimulated cultures were 0.02 (0.00–0.72), 0.02 (0.00–1.6) and 0.09 (0.01–2.1) ng/ml at 4, 8 and 24 h. As these values are low, the effect of genotype was determined in stimulated cultures, where TNFα concentrations were 1.3 (0.01–5.4), 1.4 (0.00–10.8) and 1.4 (0.01–4.5) ng/ml after 4, 8 and 24 h. As these values do not rise over time, data collected at 4 h was selected as the best measure of *de novo* synthesis (Table 1).

Carriage of the P2X4R minor alleles of rs2686387 and rs10849860 associated with an increased concentration of TNFα ($p = 0.006$ and 0.034) while rs7298368 was associated with a decrease ($p = 0.002$), supporting an effect of the gene on a TLR4-TNFα pathway. Accordingly rs2686387, which showed a weak association with HIV-SN in South Africans, is classified as an expression quantitative trait locus (eQTL) of *P2X4R* in whole blood and tibial arteries, but rs7298368 and rs10849860 are not [16]. However, the "high TNFα" allele 2 of rs2686387 aligned with resistance to HIV-SN. We therefore considered the possibility that rs2686387 may have different haplotypic associations in populations of African and European descent. These analyses were done after adjustment of the South African data because the major and minor alleles rs2686387, rs11065504, rs1718120 and rs3817190 were reversed in the African population relative to Australian Caucasians. FastPHASE derived five haplotypes in the Australian Caucasian population, none of which occurred in the South African population (Table 2). As such, the haplotypes containing rs2686387, rs7298368 and rs10849860 must be different. This leaves open the possibility that the SNPs affecting TNFα responses in Caucasians may affect neuropathy in Caucasians.

In Caucasians, one P2X4R haplotype associated with increased TNFα levels and contained rs2686387 and rs10849860. A second haplotype contained these SNP but occurred in only 2% of the population thus no association could be determined. Another haplotype which occurred in 15% of this population contained rs2686387 but not rs10849860 and did not associated with TNFα. This suggests that carriage of the minor alleles of both rs2686387 and rs10849860 are necessary to generate an effect on TNFα production.

For *CAMKK2,* carriage of minor alleles of rs11065504 and rs7314454 had small and opposing effects on TNFα concentrations, but neither SNP associated with HIV-SN in South Africans. Rs11065504 is located between *P2X4R* and *CAMKK2* so it may impact expression of either gene. Carriage of the minor allele of this SNP is reported as an eQTL in whole blood, tibial arteries and tibial nerves for P2X4R but not CaMKK2 expression [16]. The three *CAMKK2* SNPs associated with HIV-SN did not affect concentrations of TNFα detected in stimulated cultures at 4 h (Table 1), so we again considered the possibility that *CAMKK2* haplotypes may be different in populations of African and European descent. FastPHASE analyses of the Caucasian genotypes generated 18 haplotypes with frequencies > 1%. While eight of these are also observed in the South African population, the haplotypes which contain more than one minor allele associated with HIV-SN do not occur in both populations (Table 2). Overall we cannot determine whether haplotypic differences explain our failure to align the effects of individual SNPs on TNFα concentrations and HIV-SN.

A CAMKK2 haplotype found in Caucasians and South Africans at 2 and 4% (respectively) was significantly associated with reduced TNFα levels ($p = 0.04$). Accordingly this haplotype contained the minor allele of rs11065504 and the major allele of rs7314454, which were associated with low TNFα levels. However this combination of SNPs is found in five other haplotypes that had no interesting associations, so the causative SNP may lie outside the panel investigated in this study.

4. Discussion

It is plausible that the SNP tested affect aspects of TNFα production that are not modelled by *in vitro* stimulation of PBMC. Alternatively, their impact on HIV-SN may reflect another role of CaMKK2. For instance, another SNP significantly associated with HIV-SN, rs2686367, is located in the 5′UTR of *CAMKK2* [4], an area containing consensus DNA for transcription factors which regulate neuropoesis [9]. Furthermore, rs2686367 is classified as an eQTL of *CAMKK2* expression in tibial nerves [16], so this SNP may impact protein function in neuron repair.

In view of the data presented here, we cannot exclude the possibility that the strong link between *CAMKK2* polymorphisms and HIV-SN may result from interactions between the expressed protein and neurons. The high genetic variability of *P2X4R* and the effect of several SNP on TNFα concentrations leaves open the possibility that P2X4R may impact HIV-SN via a TLR4-TNFα pathway. It will be a challenge moving forward to exploit variations in haplotypes carried in different populations to identify SNPs critical to disease pathogenesis.

Conflict of interest

The authors have no conflict of interests to declare.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.humimm.2018.02.002.

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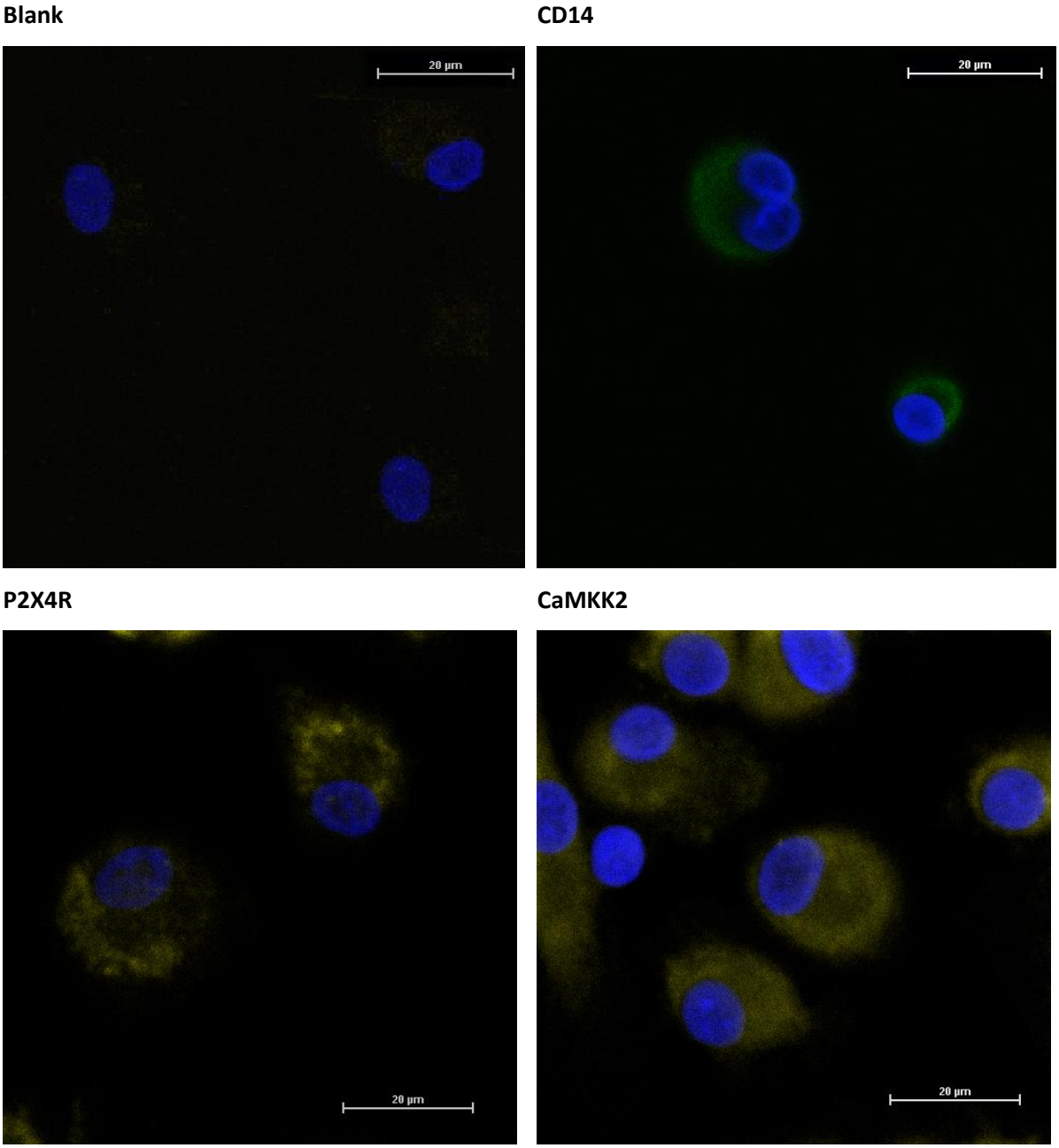
Appendix 3

SUPPLEMENT 1.



Supplementary Figure 1. CD14+ macrophages express CaMKK2 and P2X4R

Macrophages cultures isolated from human PBMC were stained with FITC labelled anti-CD14 (mouse 1:20; BioLegend, CA, USA), or unlabelled anti- CaMKK2 (goat 1:200; AbCam, Cambridge, UK) or P2X4R (goat 1:200; AbCam). The biotinylated secondary antibody donkey anti-goat IgG (AbCam, Cambridge, UK) was used at 1:100 and detected using Alexa Fluor 647 labelled Streptavidin (1:100, Life Technologies, CA, USA). Images were viewed and enhanced with Nikon A1 confocal microscope and NIS-Elements Viewer (Nikon Instruments, NY, USA).

Appendix 4

Ex-vivo expression of chemokine receptors on cells surrounding cutaneous nerves in patients with HIV-associated sensory neuropathy

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Objective: HIV-associated sensory neuropathy (HIV-SN) remains common in HIV+ individuals receiving antiretroviral therapy (ART), even though neurotoxic antiretroviral drugs (e.g. stavudine) have been phased out of use. Accumulating evidence indicates that the neuropathy is immune-mediated. We hypothesize that chemokines produced locally in the skin promote migration of macrophages and T cells into the tissue, damaging cutaneous nerves causing HIV-SN.

Design: We assessed chemokine receptor expression on infiltrating $CD14^+$ and $CD3^+$ cells around cutaneous nerves in standardized skin biopsies from HIV-SN+ patients ($n = 5$), HIV-SN− patients ($n = 9$) and healthy controls ($n = 4$).

Methods: The AIDS Clinical Trials Group Brief Peripheral Neuropathy Screen was used to assess Indonesian HIV+ patients receiving ART without stavudine (case definition: bilateral presence of at least one symptom and at least one sign of neuropathy). Distal leg skin biopsies were stained to visualize chemokine receptors (CCR2, CCR5, CXCR3, CXCR4, CX3CR1), infiltrating $CD3^+$ and $CD14^+$ cells, and protein-gene-product 9.5 on nerves, using immunohistochemistry and 4-colour confocal microscopy.

Results: Intraepidermal nerve fibre density was variable in patients without HIV-SN and generally lower in those with HIV-SN. CX3CR1 was more evident on $CD14^+$ cells whereas CCR2, CCR5, CXCR3 and CXCR4 were more common on $CD3^+$ cells. Expression of CX3CR1, CCR2 and CCR5 was more common in HIV-SN+ patients than those without HIV-SN. CXCR3 and CXCR4 were upregulated in all HIV+ patients, compared with healthy controls.

Conclusion: Inflammatory macrophages expressing CX3CR1 and T cells expressing CCR2 and CCR5 may participate in peripheral nerve damage leading to HIV-SN in HIV+ patients treated without stavudine. Further characterization of these cells is warranted.
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Keywords: $CD14^+$ cells, $CD3^+$ cells, chemokine receptors, HIV-sensory neuropathy

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Introduction

HIV-associated sensory neuropathy (HIV-SN) is a common neurologic manifestation of HIV and its treatment. Historically, neuropathy has been described in about 30% of treatment-naive patients with advanced HIV disease [1,2], and about 60% of patients on antiretroviral therapy (ART) regimens that included zalcitabine, didanosine or stavudine [3,4]. It is now accepted that these drugs are neurotoxic. Despite patients now starting treatment earlier and without known neurotoxic antiretroviral agents, HIV-SN remains a problem [5–7].

A pathological hallmark of HIV-SN is distal degeneration of long axons in a 'dying back' pattern, which is associated with reduced intraepidermal nerve fibre density (IENFD), nerve fibre swelling and mononuclear cell infiltration [8,9]. HIV-infected and uninfected activated macrophages can infiltrate peripheral nerves, dorsal root ganglia (DRG) [10] and/or tissues adjacent to peripheral nerves, and release cytokines such as tumour necrosis factor alpha (TNF-α), interferon-gamma (IFN-γ) and interleukin (IL)-1 and/or IL-17, which can cause axonal degeneration [11]. TNF-α injected into rat sciatic nerve stimulated neuropathic pain behaviour [12] and TNF-α mRNA levels were increased in peripheral nerve tissue from AIDS patients [13]. Furthermore, genetic association studies have linked polymorphisms and haplotypes from *TNF* and surrounding genes with increased risk of HIV-SN in Africans [14], Asians and Caucasians [15].

Chemokines produced in cutaneous tissues can bind to their receptors expressed on neuronal and inflammatory cells, initiating damage to the nerves. CCR5 and CXCR4 are co-receptors supporting HIV-1 entry [16]. CCR1, CCR2, CCR4, CCR5, CXCR4 and CX3CR1 are expressed on subpopulations of sensory neurons and their axons [17,18]. CCR2, CCR5 and CXCR4 are upregulated in primary sensory neurons and adjacent nonneuronal cells following peripheral nerve injury in animal models [19,20]. HIV-1 envelope glycoprotein 120 (gp120) may bind CCR5 and/or CXCR4 on nerve cells causing direct axonal damage [21], but there is no evidence of HIV infecting peripheral nerves in humans. In primary DRG cultures, gp120 mediated neuronal toxicity via TNF-α/TNF receptor (TNFR)-1 signalling [22]. CXCR4 and CCR5 ligation by CXCL12 and CCL5 (respectively) mimicked neurotoxicity induced by gp120 [22]. Administration of gp120 into rat sciatic nerve, upregulated CCL2/CCR2 and triggered hypernociception [23]. CCR2 expression was upregulated on primary sensory neurons and Schwann cells after peripheral nerve injury [24], and CCR2-knockout mice showed reduced pain behaviour following partial ligation of sciatic nerves [25]. CX3CL1 can recruit macrophage expressing CX3CR1. CX3CR1 was upregulated on spinal microglia and DRG glial satellite cells following peripheral nerve injury [26], and CX3CR1-deficient mice showed reduced neuropathic pain behaviour [27].

There is a reasonable consensus linking HIV-SN with a reduced IENFD [28,29], but no studies have addressed whether HIV-SN is associated with critical chemokine signalling pathways. Here we present evidence on the ex-vivo expression of chemokine receptors on infiltrating $CD14^+$ and $CD3^+$ cells around nerves in skin biopsies from HIV+ patients exposed to modern ART regimens that excluded the known neurotoxic agents. We believe we are the first to investigate chemokine-signalling pathways and IENFD in this context. These results will enhance the knowledge of the underlying pathogenesis of HIV-SN in the modern era of HIV care.

Materials and methods

Patients and controls

HIV+ patients treated at Cipto Mangunkusumo Hospital, Jakarta, Indonesia, were screened for sensory neuropathy using the AIDS Clinical Trials Group Brief Peripheral Neuropathy Screen (ACTG-BPNS), a validated tool based on detection of clinical signs (reduced/absent ankle reflexes or absent/diminished vibration sense) and symptoms (pain, aching, burning, pins and needles or numbness) of neuropathy [30]. We used the standard ACTG-BPNS case definition for HIV-SN: bilateral presence of at least one clinical sign and at least one symptom. Patients had received ART for at least 12 months (median: 4.7 years; range: 1 – 12) and had never received stavudine. Biopsies from 14 HIV+ participants (HIV-SN+, $n = 5$, HIV-SN−, $n = 9$) were used. Control biopsies were obtained from Asian volunteers from Jakarta (male volunteer, $n = 1$) and Curtin University, Australia (women, $n = 3$). All donors are described in Supplementary Table 1, http://links.lww.com/QAD/B202. The study was approved by the Ethics Committee of the Faculty of Medicine, University of Indonesia (579/UN2.F1/ETIK/2014) and validated by Curtin University (HR210–2015). All participants gave written informed consent.

Sample collection and preservation

Local anaesthetic was injected and 3 mm punch skin biopsies were collected ∼10 cm above the lateral malleolous on the distal leg under sterile conditions. Biopsies were placed in 4% paraformaldehyde-lysine-periodate fixative for 12–24 h at 4 °C before transfer to glycerol-based cryoprotectant (20% glycerol, 20% 0.4 mol/l Sorrenson's phosphate buffer and 60% dH_2O) for storage at -20 °C. Biopsies were cut perpendicular to the epidermal surface on a freeze cryostat sliding microtome (Microm HM550; Thermo Fisher Scientific, Waltham, Massachusetts, USA) set at 50 μmol/l , placed

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into antifreeze (33% glycerol, 33% ethylene glycol, 10% 2× phosphate buffer and dH_2O) and stored at −20 °C for immunochemistry (IHC).

Immunochemical staining

Staining was performed in 24-well plates. Sections were bleached with 0.25% potassium permanganate (15 min, room temperature), washed with 1 ml Tris-buffered saline (TBS) containing 0.1% Triton-X, and treated with 5% oxalic acid (2 min). Sections were then blocked with Image-iT FX Signal Enhancer (Invitrogen, Carlsbad, California, USA) for 30 min. This solution was removed before the addition of primary antibodies.

To identify chemokine receptors on $CD14^+$ cells, sections were treated (overnight, 4 °C) with mouse-monoclonal IgG antibodies against CCR2, CCR5, CXCR3 or CXCR4 (5 μg/ml; R&D Systems, Minneapolis, Minnesota, USA) or CX3CR1 (10 μg/ml; Biolegend, San Diego, California, USA), biotinylated polyclonal sheep IgG anti-CD14 (R&D systems) and polyclonal rabbit IgG anti-protein-gene-product 9.5 (PGP9.5; 2 μg/ml) to detect nerves (Abcam, Cambridge, Massachusetts, USA). Sections were washed five times with TBS, followed by six 1-h washes before adding secondary antibodies; goat anti-mouse IgG FITC (20 μg/ml), donkey anti-rabbit IgG Dylight (5 μg/ml; Abcam) and AlexaFluor fluorochrome streptavidin (20 μg/ml; Invitrogen). Secondary antibodies were diluted in 2% donkey, goat and human serum and applied overnight at 4 °C.

To identify chemokine receptors on $CD3^+$ cells, sections were treated (overnight, 4 °C) with polyclonal goat IgG against CCR2, CCR5, CXCR4 (20 μg/ml) or CXCR3 (10 μg/ml), mouse-polyclonal IgG anti-CD3 (10 μg/ml; Novus Biologicals, Littleton, Colorado, USA) and anti PGP9.5 (as above). Sections were washed as described above and treated with biotinylated donkey anti-goat IgG (20 μg/ml; Abcam). Sections were blocked with 1% goat serum for 30 min before addition of AlexaFluor fluorochrome streptavidin (20 μg/ml; Invitrogen), goat anti-mouse IgG FITC (20 μg/ml) and donkey anti-rabbit IgG (5 μg/ml; Abcam). Secondary antibodies were diluted in 1% goat serum and 2% donkey serum, and applied overnight at 4 °C. Stained sections were washed six times, incubated with 4′,6-diamidino2-phylindole, diyhdrochloride (10 min; Invitrogen), washed twice with TBS, mounted using Shandon Immumount (Thermo Fisher Scientific) and coverslips (#1.5; Proscitech, Queensland, Australia) before viewing. One section, stained only with secondary antibodies was included in each run as a negative control.

Visualization of sections using confocal microscopy

Images were acquired using an inverted Nikon A1+ confocal microscope with NIS-Elements confocal software (Nikon Instruments, Tokyo, Japan). Images were collected at digital scan resolution 0.62 μm/pixel, pixel dwell 4.8 with 1024 resolution using a 20× Plan Apo dry objective (N.A. 0.75). Sequential laser scanning was performed using four lasers; 405 nm (450/50 filter), 488 nm (525/50 filter), 561 nm (595/50 filter) and 640 nm (700/75 filter) to view nuclei, chemokine receptors, nerve fibres and $CD14^+$ or $CD3^+$ cells, respectively. The position of the top and bottom of the image was recorded before multiple images were taken in a z-series, collected according to Nyquist criteria. Equivalent thresholds were applied across images to visualize nerves (white), chemokine receptors (red) and $CD14^+$ or $CD3^+$ (green). Chemokine receptors co-located with either $CD14^+$ or $CD3^+$ cells appeared yellow.

Intraepidermal nerve fibre density

NIS-Elements Advanced Research software (Nikon Instruments) was used to acquire three 0.5 mm^2 sections per biopsy (only two samples were available for one participant). Sections were coded and nerve fibres were counted by six investigators using standardized rules for IENFD quantification [31]. In brief, single IENF crossing the dermal–epidermal junction is counted with secondary branching excluded from quantification. The average count across the three sections per biopsy was multiplied by 2 to generate IENFD per square millimeter area of skin for each participant (Supplementary Figure 1, http://links.lww.com/QAD/B202).

We computed Light's κ for exact agreement (zero tolerance) between the six raters, treating rating as a weighted variable and using squared distance. A bootstrap 95% confidence interval (CI; $n = 1000$ resamples) was calculated using the bias-corrected and accelerated bootstrap method. The analysis yielded a Light's $\kappa = 0.79$ (95% CI 0.61–0.91); the point estimate indicating strong inter-rater agreement, with the CI indicating moderate-to-strong agreement [32].

Results

Intraepidermal nerve fibre density was generally reduced in HIV-SN+ patients

HIV-SN+ ($n = 5$) and HIV-SN− ($n = 9$) patients were matched for age, height, time on ART and $CD4^+$ T-cell counts (Supplementary Table 1, http://links.lww.com/QAD/B202). Healthy controls ($n = 4$) were also matched with the patients by age and height. All donors were of South East Asian ancestry. Figure 1 shows confocal images from two healthy controls (a and b), two HIV-SN− patients (c and d) and two HIV-SN+ patients (e and f) selected to represent the range seen in each group. Median (range) IENFD per square millimeter field were 11.2 (5.8–15.2), 5.8 (1.4–14.0) and 3.0 (0.8–9.7) in healthy controls, HIV-SN− and HIV-SN+ groups, respectively. The IENFD

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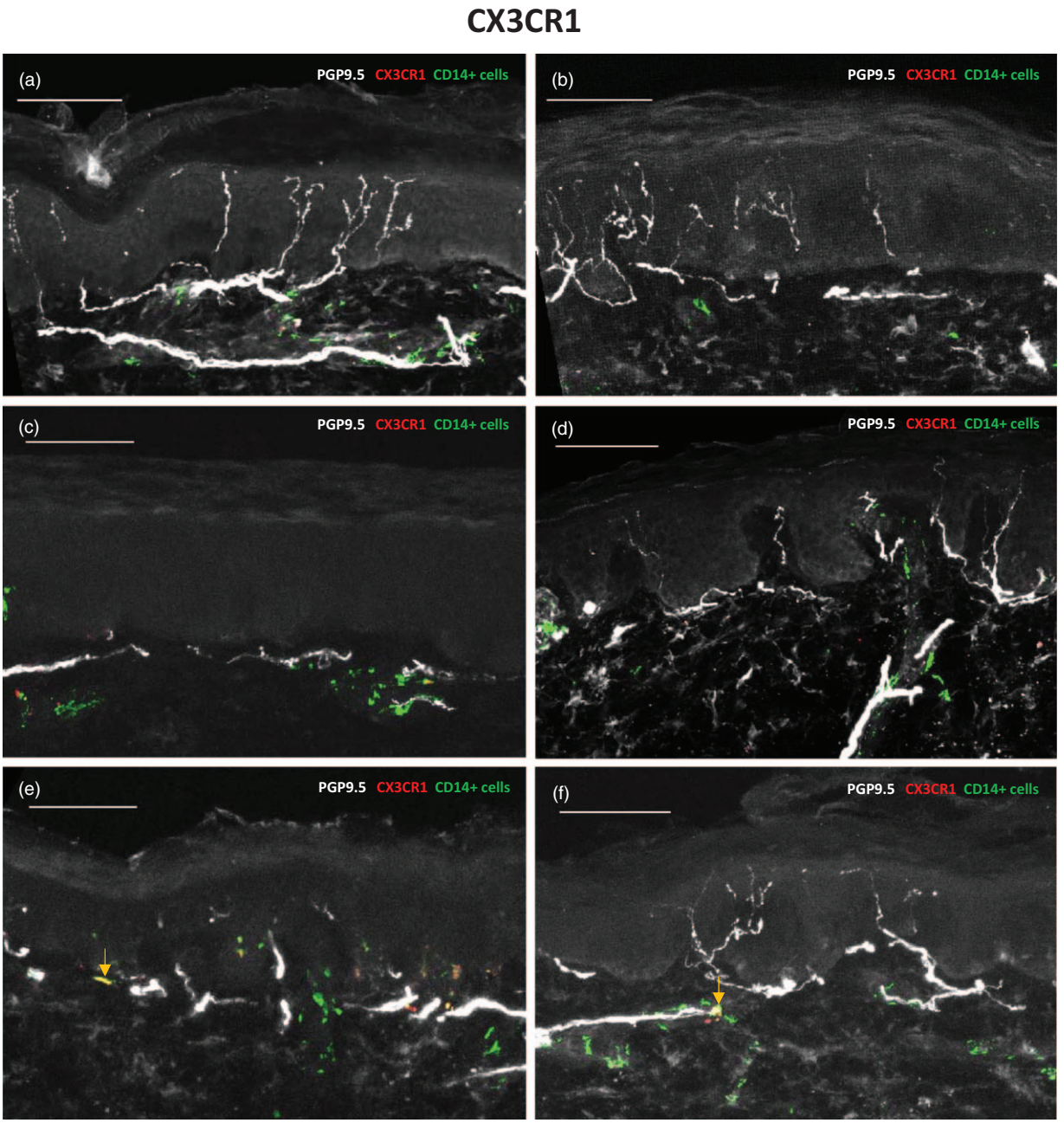

Fig. 1. Representative confocal images showing expression of CX3CR1 and CD14 in skin from two healthy controls (a and b), HIV-SN− (c and d) and HIV-SN+ (e and f) patients. Abundant thin intraepidermal nerve fibres run from the dermis innervating the basement membrane and epidermis in healthy controls' skin sections. The median nerve count (IEFND) was 11.2 (range: 5.8–15.2) fibres per square millimeter skin area. IENFD was variable in HIV-SN− sections (5.8 [1.3–14.0]) and slightly lower in HIV-SN+ (3.0 [0.8–9.7]). CX3CR1 was rare in healthy controls' skin sections and minimally expressed in skin sections from patients. However, CX3CR1 expression was closely associated with epidermal nerves in HIV-SN+ sections, and co-localized with $CD14^+$ cells (e and f; yellow arrows). The white lines represent 100 μm.

tended to be reduced in HIV-SN+ patients compared with healthy controls, but the study was not powered to find a significant difference (Supplementary Tables 1 and 2; Supplementary Figure 1, http://links.lww.com/QAD/B202). A reduction in the length of nerve fibres within the epidermis of HIV-SN+ patients was common. $CD14^+$ macrophages were visible in sections from HIV+ patients. Many were adjacent to blood vessels, scattered within cutaneous tissue or adjacent nerve fibres.

CX3CR1 was expressed on $CD14^+$ cells adjacent to peripheral nerves in HIV-SN+ patients

Sections were stained to visualize expression of CX3CR1 on infiltrating $CD14^+$ cells (Fig. 1). CX3CR1

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expression was extremely rare in the three sections from healthy controls (a and b). They were also rare in sections from three HIV-SN− patients (e.g. c and d). Few CX3CR1^{+} cells were seen in two of three sections from HIV-SN+ patients (e and f). In all sections from HIV-SN+ patients, CX3CR1 was co-located with CD14 (yellow arrows) and was closely associated with the subepidermal nerve plexi [Fig. 1(e and f)]. This is consistent with a role for the receptor in HIV-SN.

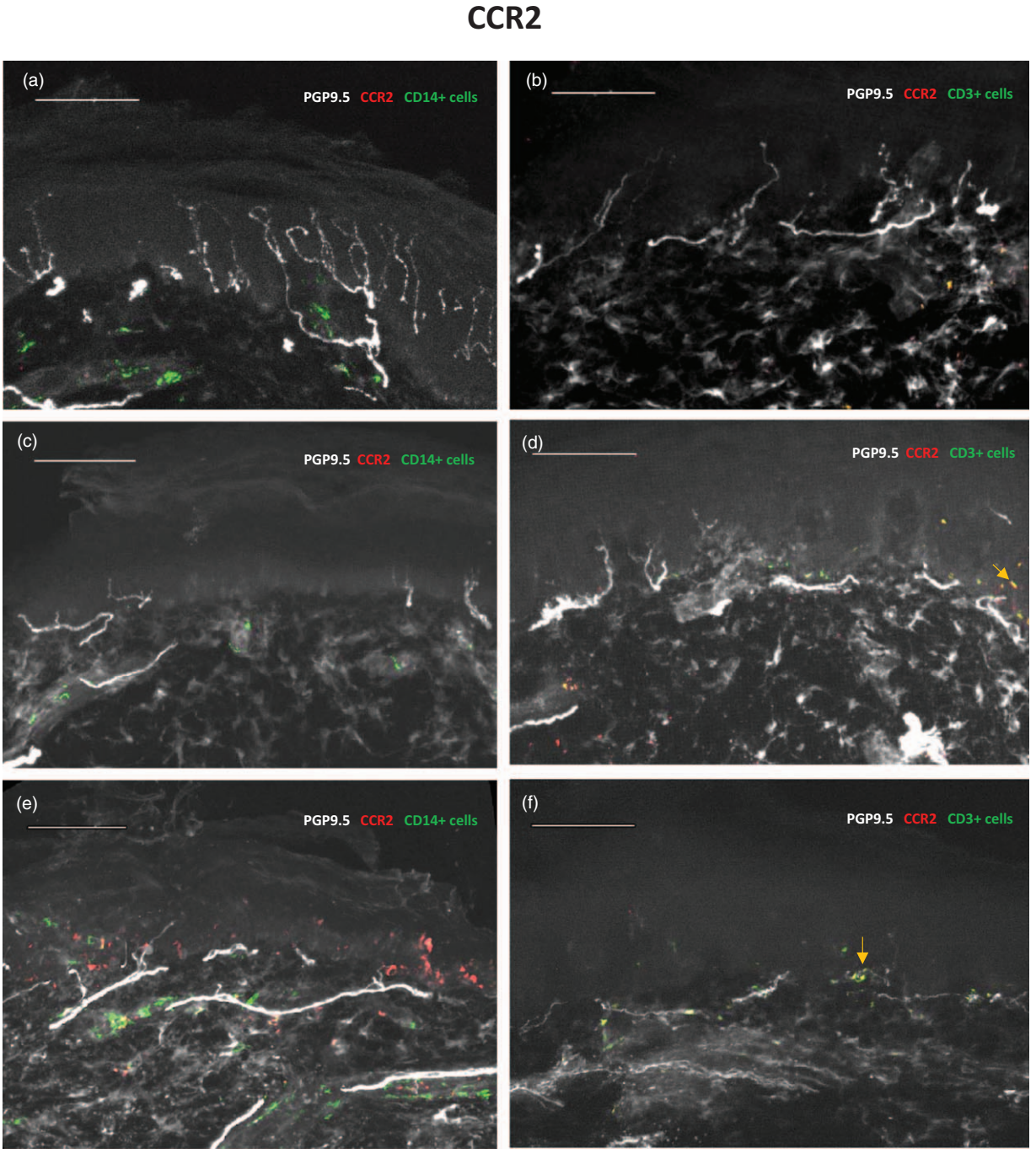

Fig. 2. Representative confocal images showing expression of CCR2 with CD14 (left) or CD3 (right) in skin from healthy controls (a and b), HIV-SN− (c and d) and HIV-SN+ (e and f) patients. CCR2 expression was rare in healthy controls. Two of eight stained sections from HIV-SN− patients had detectable CCR2^{+} cells, whereas seven of seven sections from five HIV-SN+ cases displayed CCR2. Some CCR2^{+} cells were located close to nerves. CCR2 was rarely expressed on CD14^{+} cells (e), but was seen on CD3^{+} cells (d and f; yellow arrows).

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CCR2 is upregulated in HIV-SN+ skin and predominantly expressed by CD3$^+$ cells

Sections were stained to visualize CCR2 on infiltrating CD14$^+$ or CD3$^+$ cells (Fig. 2). Very few CCR2$^+$ cells were evident in healthy controls (e.g. a and b). Two of eight stained sections from HIV-SN− cases displayed detectable CCR2$^+$ cells (e.g. Fig. 2d), whereas all seven sections from five HIV-SN+ cases displayed CCR2 expression (e.g. e and f). The HIV-SN− patient expressing CCR2 most clearly (patient 11; not shown) had the lowest IENFD and a case review uncovered a history of Stevens Johnson Syndrome – an inflammation of the skin. The patient was excluded from further IHC. Most CCR2 was co-located with CD3 (d and f, yellow arrows). CD14$^+$ cells were visible but rarely expressed CCR2 (a, c and e).

CCR5 is upregulated and associated with peripheral nerves in HIV-SN+ skin sections

Sections were also stained to visualize expression of CCR5 on infiltrating CD14$^+$ or CD3$^+$ cells (Fig. 3). CCR5$^+$ cells were very rare in healthy controls (a and b). Isolated positive cells were seen in two of five samples from HIV-SN− (c and d) and five of five samples from HIV-SN+ patients, with some cells located close to nerve fibres (e and f). CCR5 was co-located with CD3 (yellow arrows, d and f), but not CD14 (a, c and e).

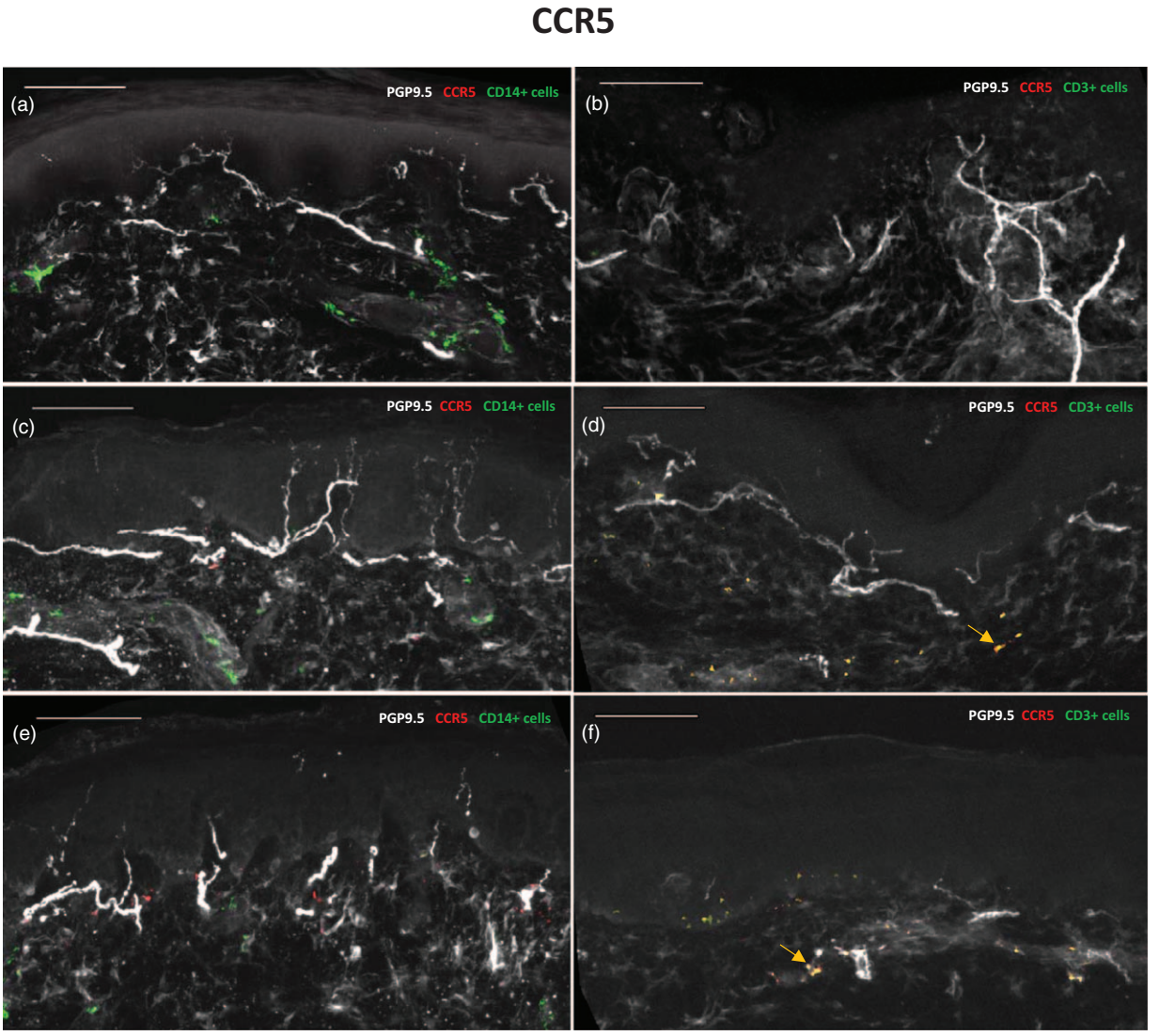

Fig. 3. Representative confocal images showing expression of CCR5 with CD14 (left) or CD3 (right) in skin from healthy controls (a and b), HIV-SN− (c and d) and HIV-SN+ (e and f) patients. CCR5$^+$ cells were rare in sections from healthy controls (a and b), but expression was up-regulated in sections from HIV+ patients; HIV-SN− (two of five sections) and HIV-SN+ (six of six sections). CCR5 was predominantly co-localized with CD3 (d and f; yellow arrows). Some CCR5$^+$ cells were located close to nerve fibres in sections from HIV-SN+ patients (e and f).

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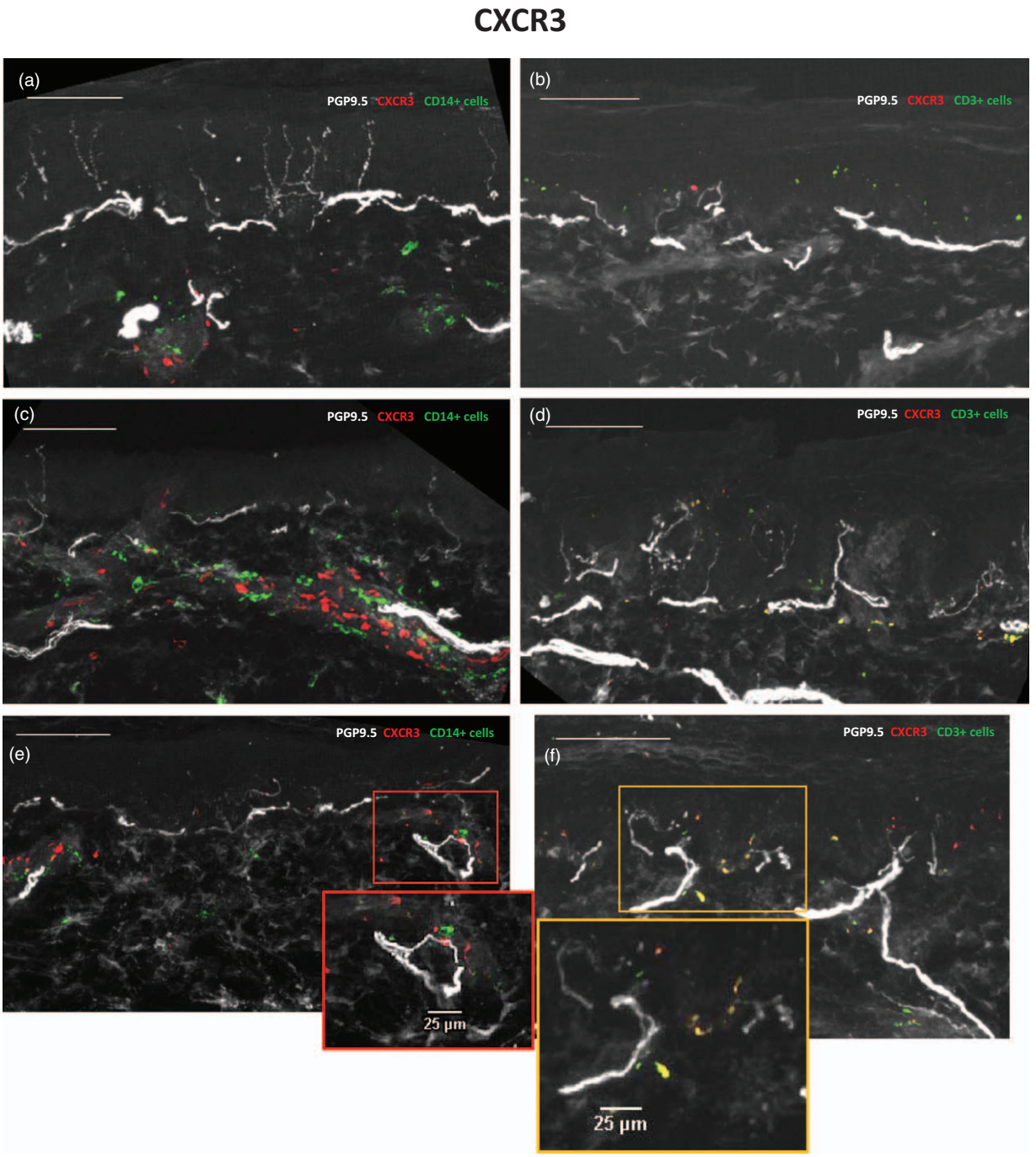

Fig. 4. Representative confocal images showing expression of CXCR3 with CD14 (left) or CD3 (right) in skin from healthy controls (a and b), HIV-SN− (c and d) and HIV-SN+ (e and f) patients. CXCR3 was rare in healthy controls, mostly surrounding dermal blood vessels. CXCR3 was highly expressed in sections from all HIV+ patients, and most commonly co-located with CD3 (d and f; yellow arrows). An expanded red box (e) highlights the close proximity of $CD14^+$ (green) and $CXCR3^+$ (red) cells on the nerve. A yellow box (f) shows the co-localization of CD3 and CXCR3 (yellow) adhering to a cutaneous nerve in a HIV-SN+ section.

CXCR3 was expressed by scattered $CD3^+$ cells in all HIV+ patients

$CXCR3^+$ cells were visible in blood vessels present in some sections from all groups. $CXCR3^+$ cells located closely with $CD14^+$ cells but the two markers did not co-stain (Fig. 4a, c and e). In addition, variable numbers of $CD3^+$ $CXCR3^+$ cells were seen scattered in the tissues, so that some were adjacent to nerves in all eight sections from HIV-SN− and three sections from HIV-SN+ patients. Fewer positive cells were seen in samples

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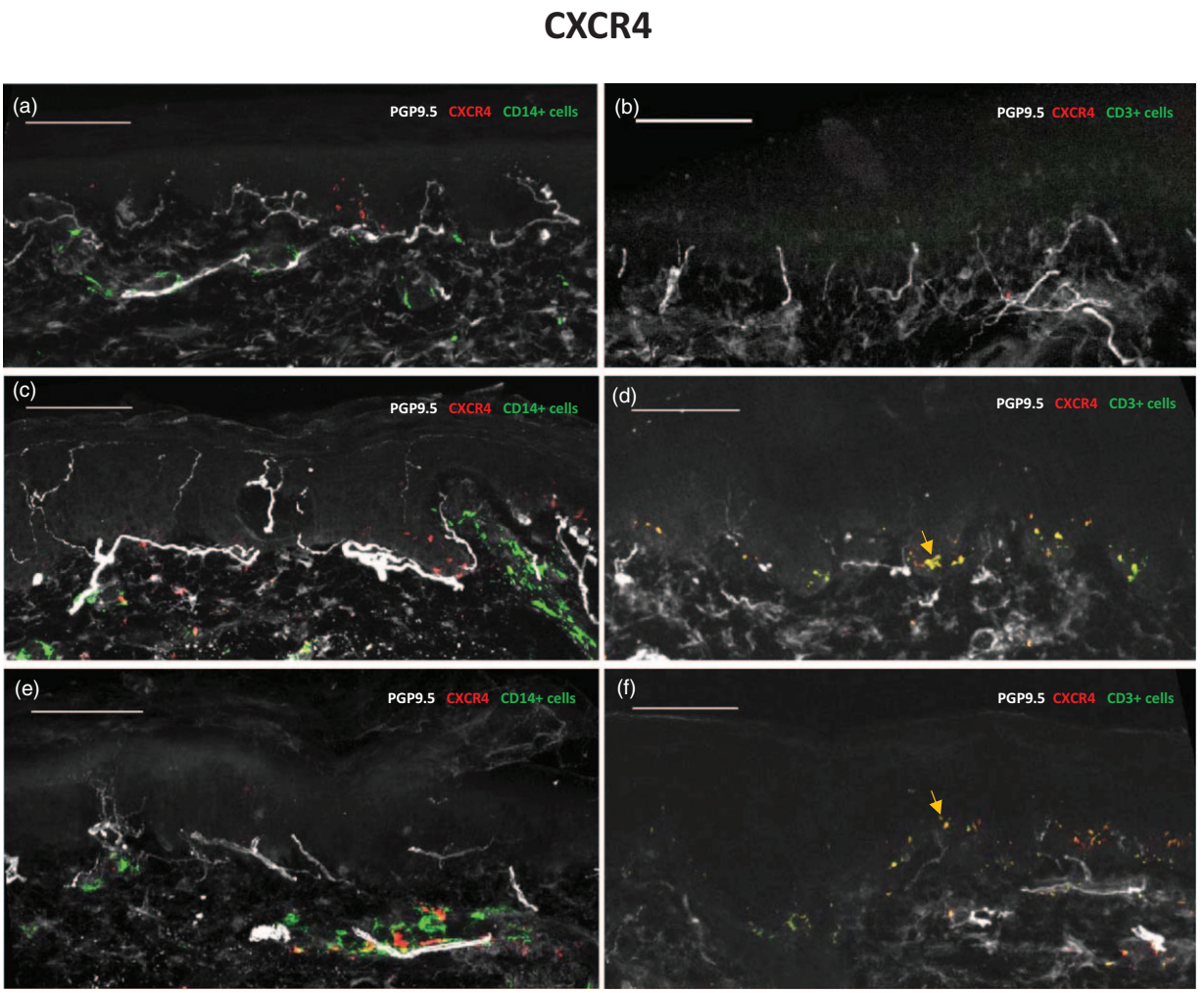

Fig. 5. Representative confocal images showing expression of CXCR4 with CD14 (left) or CD3 (right) in skin from healthy controls (a and b), HIV-SN− (c and d) and HIV-SN+ (e and f) patients. CXCR4 was upregulated in the epidermis and the dermis of HIV+ patients, with mixed patterns of expression (c–f). CXCR4 was rarely expressed on $CD14^+$ cells (a, c and e) but was seen on $CD3^+$ cells (d and f; yellow arrows).

from healthy controls, so expression of this marker may be a consequence of HIV infection.

$CXCR4^+$ $CD3^+$ cells were seen along the epidermis and the dermis in HIV+ patients

$CXCR4^+$ cells were seen in some but not all sections from healthy controls [Fig. 5(a and b)]. CXCR4 was expressed in all six sections from HIV-SN− patients (c and d) and three HIV-SN+ patients (e and f) and was distributed along the epidermis (c and f) or adjacent to blood vessels (e). Some larger cells expressed CXCR4 without $CD14^+$ (e). CXCR4 was expressed on a subset of $CD3^+$ cells in the dermis (d and f). This may reflect HIV disease rather than HIV-SN.

Discussion

We have developed a protocol that identifies cells and receptors that could contribute to the damage of small nerve fibres and form the basis of HIV-SN. The compilation of images into z-series allowed us to follow individual nerves as if they were distributed in the tissue in just two dimensions. Counts made by multiple observers blinded to the disease phenotype provide reliable quantification of IENFD (Supplementary Figure 1, http://links.lww.com/QAD/B202). The median IENFD at the distal leg of normal controls was 11.2/mm^2. This is consistent with an earlier study with the reference range of $13.8 \pm 6.7/mm^2$ [33]. IENFD was generally reduced in HIV-SN+ patients and variable in those without HIV-SN. The wide range of nerve fibre densities in HIV-SN− patients may reflect early lesions not detected by the ACTG-BPNS. A recent longitudinal study of 150 Thai HIV+ individuals found that IENFD measurements did not distinguish individuals with HIV-SN or signal the onset of neuropathic signs/symptoms [34]. However, IENFD decreases with increasing age [35]. Here all donors were 25–47 years old so any effect from age is likely to be minimal. We were also unable to assess associations with

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genotype [14,36], but all participants were of South East Asian descent.

In addition to a reduction in the number of nerves in the epidermis, our methods demonstrate a reduction in cutaneous nerve fibre length in adults with HIV-SN (Fig. 1e) and in some HIV-SN− cases (Fig. 1c). It is unlikely that the loss of nerves reflects direct infection by HIV [9]. However, HIV-infected macrophages may have a primary role in nerve damage or may accumulate in response to debris from destroyed axons [37]. Our results show that $CD14^+$ macrophages were visible in all sections from all HIV+ patients and some healthy controls. These $CD14^+$ macrophages may release pro-inflammatory cytokines causing axonal and DRG neuronal injury [38,39]. A recent study has linked the loss of IENFD with increased recruitment of macrophages to DRG in Simian Immunodeficiency Virus-infected macaques [40]. We show macrophages adjacent to blood vessels, scattered within cutaneous tissues and adjacent to nerve fibres. This distribution is consistent with their extravasation and migration towards the nerves, a pattern observed in other chronic inflammatory neuropathies [41]. These $CD14^+$ macrophages did not express CCR2, CCR5, CXCR3 or CXCR4 but did express CX3CR1.

$CX3CR1^+$ monocyte/macrophage are expressed in low levels and are recruited in healing tissues [42]. CX3CL1/CX3CR1 signalling is implicated in the development of neuropathic pain in animal models [27,43–45]. Our results show CX3CR1 was minimally expressed, but $CX3CR1^+$ $CD14^+$ macrophages were seen near the residual nerves in sections from HIV-SN+ patients. This supports a study showing increased expression of CX3CR1 by macrophages in the sciatic nerve proximal to a site of mechanical injury and in the corresponding DRG [26]. Furthermore, in a spinal nerve ligation model, CX3CR1 expression was upregulated in spinal microglia, whilst membrane-bound levels of CX3CL1 were reduced [43]. The cleavage of CX3CL1 after nerve injury may initiate activation of the low-affinity purinergic P2X7 receptor, leading to the release of the lysosomal cysteine protease cathepsin S from microglia. This may mediate neural–glial interaction and neuropathic pain behavior [46]. Accordingly, the gene encoding the P2X7 receptor, *P2X7R*, lies in a region of linkage disequilibrium with upstream genes *P2X4R* and *CAMKK2*. Single nucleotide polymorphisms and haplotypes within this block of genes were associated with HIV-SN in South African HIV+ individuals [47]. Other studies suggest downstream mechanisms of CX3CR1 via p38 mitogen-activated protein kinases [43] or extracellular signal-regulated protein kinase 5 [48] may activate microglia after nerve injury. Blockade or knockout of CX3CR1 impaired neuropathic pain behaviours and reduced hypersensitivity to thermal stimuli following peripheral nerve injury in animal models [27,43,44]. These considerations support our evidence that CX3CR1 could have a role in HIV-SN.

Our results show CCR2, CCR5, CXCR3 and CXCR4 were co-localized with CD3 but not CD14. T lymphocytes were observed in nerves obtained from patients with inflammatory neuropathies [49]. An immunocytochemical study investigating mononuclear cells in sural nerve biopsies from 42 HIV− and HIV+ patients with various types of peripheral neuropathy found that 72% of infiltrating mononuclear cells were $CD3^+$ [50]. T cell infiltration into the spinal dorsal horn after nerve injury was also implicated in the development of pain-like hypersensitivity in rats [51]. CCR2 is critical in recruiting T cells in responses to axonal injury of the central nervous system [52]. CCL2/CCR2 expression were upregulated by primary sensory neurons and Schwann cells after a sciatic nerve constriction injury [24]. CCL2/CCR2 was also upregulated by gp120 injected into rat sciatic nerve. This paralleled the development of mechanical hypernociception [23]. Accordingly, we found CCR2 was upregulated and expressed in five of five HIV-SN+ skin sections.

CCR5 and CXCR4 are co-receptors for HIV. CXCR4 was expressed in all HIV+ skin sections whilst CCR5 was more evident in skin sections from patients with sensory neuropathy, with some positive cells located near nerve fibres. In a study of chemokine receptors in HIV/gp120-induced neurotoxicity based on mixed neuronal–glial cerebrocortical cultures, gp120 utilized CCR5, CXCR4 or both to cause neurotoxicity [53]. Interestingly, CCL4 and CCL5 (ligands of CCR5) can inhibit gp120-induced neuronal death, whilst CXCL12 (the ligand of CXCR4) could alone be neurotoxic. Moreover, CCR5 ligands could inhibit CXCR4/CXCL12-induced neurotoxicity [53]. Hence our finding linking CCR5 with HIV-SN may place the receptor in a complex cascade. For example; the binding of gp120 to CXCR4 on Schwann cells can cause the release of CCL5. CCL5 can stimulate the production of TNF-α by neuronal cells in DRG, leading to TNFR1-mediated neurotoxicity [22].

CXCR3 co-localized with CD3 in all HIV+ patients. CXCR3 plays role in the adaptive immune response to inflammation and viral infection [54]. Some $CXCR3^+$ cells were located near damaged nerves. In patients with diabetic neuropathy, quantitative polymerase chain reaction and flow cytometric analyses showed that $CXCR3^+$ $CD8^+$ T cells were recruited and infiltrated into affected tissues [55]. CXCR3 ligands, CXCL9, CXCL10 and CXCL11, released from Schwann cells can further recruit $CXCR3^+$ $CD8^+$ T cell into sites of peripheral neuropathy [56]. Furthermore, Schwann cells can stimulate $CD8^+$ T cells to release TNF-α and programmed death-ligand 1 (PD-1) leading to neuronal apoptosis [55]. In foetal neuronal cultures, ligation of CXCR3 and CXCL10 can increase intracellular calcium, which in turn increases membrane permeability and cytochrome c release. This activates caspase-9, which activates caspase-3, ultimately leading to neuronal

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apoptosis [56]. Caspase-3-dependent neuronal apoptosis cascades have been demonstrated in studies of gp120-induced neurotoxicity [22,57].

Our study has some limitations. First, tissue samples were small, and sampling error remains a possible issue as we did not scan the entire biopsy. Second, we used one validated but simple clinical tool to distinguish patients with structural changes to the cutaneous nerves (HIV-SN+) from those without (HIV-SN−). We cannot exclude the possibility that some of our 'SN free' HIV+ patients may have had early, sub-clinical peripheral nerve lesions that were not detected by this tool. Third, the presence of chemokine receptors in skin tissues does not prove that their signalling is critical. However, we have linked $CD14^+$ macrophages expressing CX3CR1, and $CD3^+$ T-cells expressing CCR2 and/or CCR5 with HIV-SN in skin sections from HIV+ individuals. The cells may have a role in the loss of nerves (evident from the IENFD) or may impact upon nerve function creating the characteristic signs and symptoms of HIV-SN (numbness, tingling, pain, reduced vibration sense, etc.). Expression of CXCR3 and CXCR4 was linked with HIV disease as these receptors were found in all sections from patients. Further investigation is needed with longitudinal studies including samples collected during earlier phases of HIV-SN.

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Conflicts of interest

There are no conflicts of interest.

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SUPPLEMENTS

Table 1; Characteristics of patients and healthy controls (HC)

Donor	BPNST[a]	Gender	Age (years)	Height (cm)	Time on ART (years)	Last CD4 count (cell/uL)	IEFND[b] (per mm^2)
Patient 1	HIV-SN+	Female	33	158	7	406	1.6
Patient 2	HIV-SN+	Female	33	167	7.5	729	9.7
Patient 3	HIV-SN+	Male	34	175	5	714	3.0
Patient 4	HIV-SN+	Male	47	167	6.8	284	4.6
Patient 5	HIV-SN+	Male	45	167	3.5	300	0.8
Patient 6	HIV-SN-	Male	38	179	8.7	626	6.7
Patient 7	HIV-SN-	Female	41	150	1	435	6.6
Patient 8	HIV-SN-	Male	35	165	12	693	5.4
Patient 9	HIV-SN-	Female	32	165	3.6	385	9.0
Patient 10	HIV-SN-	Male	44	171	2.8	84	4.0
Patient 11	HIV-SN-	Male	36	168	1	566	1.3
Patient 12	HIV-SN-	Male	25	165	1.6	653	14.0
Patient 13	HIV-SN-	Male	34	166	2.2	386	5.2
Patient 14	HIV-SN-	Female	41	150	1	448	5.8
HC1	-	Female	28	158	-	-	-
HC2	-	Female	33	162	-	-	11.2
HC3	-	Female	33	151	-	-	15.2
HC4	-	Male	37	169	-	-	5.8

[a] Brief Peripheral Neuropathy Screening Tool

[b] Inter Epithelial Nerve Fibre Density

Table 2; Summary of donor characteristics

(BPNST)	N value	Male (*n*)	Age (years)	Height (cm)	Time on ART (years)	Last CD4 (cell/uL)	IEFND (per mm^2)
HIV-SN+	5	3	34 (33-47)	167 (158-175)	6.8 (3.5-7.5)	406 (284-729)	3.0 (0.8 – 9.7)
HIV-SN-	9	6	36 (25-44)	165 (150-179)	2.2 (1.0-12)	448 (84-693)	5.8 (1.3 – 14.0)
HC	4	1	33 (28-37)	160 (151-169)	n/a	n/a	11.2 (5.8 – 15.2)
		p=1.0[a]	*p*=0.84[b]	*p*=0.50 [b]	*p*=0.14 [b]	*p*=1.0 [b]	*p*=0.19 [b]

Results are presented as median (range); n/a not applicable

[a] Fisher's exact test (HIV-SN+ *versus* HIV-SN-), [b] Mann-Whitney test (HIV-SN+ *versus* HIV-SN-)

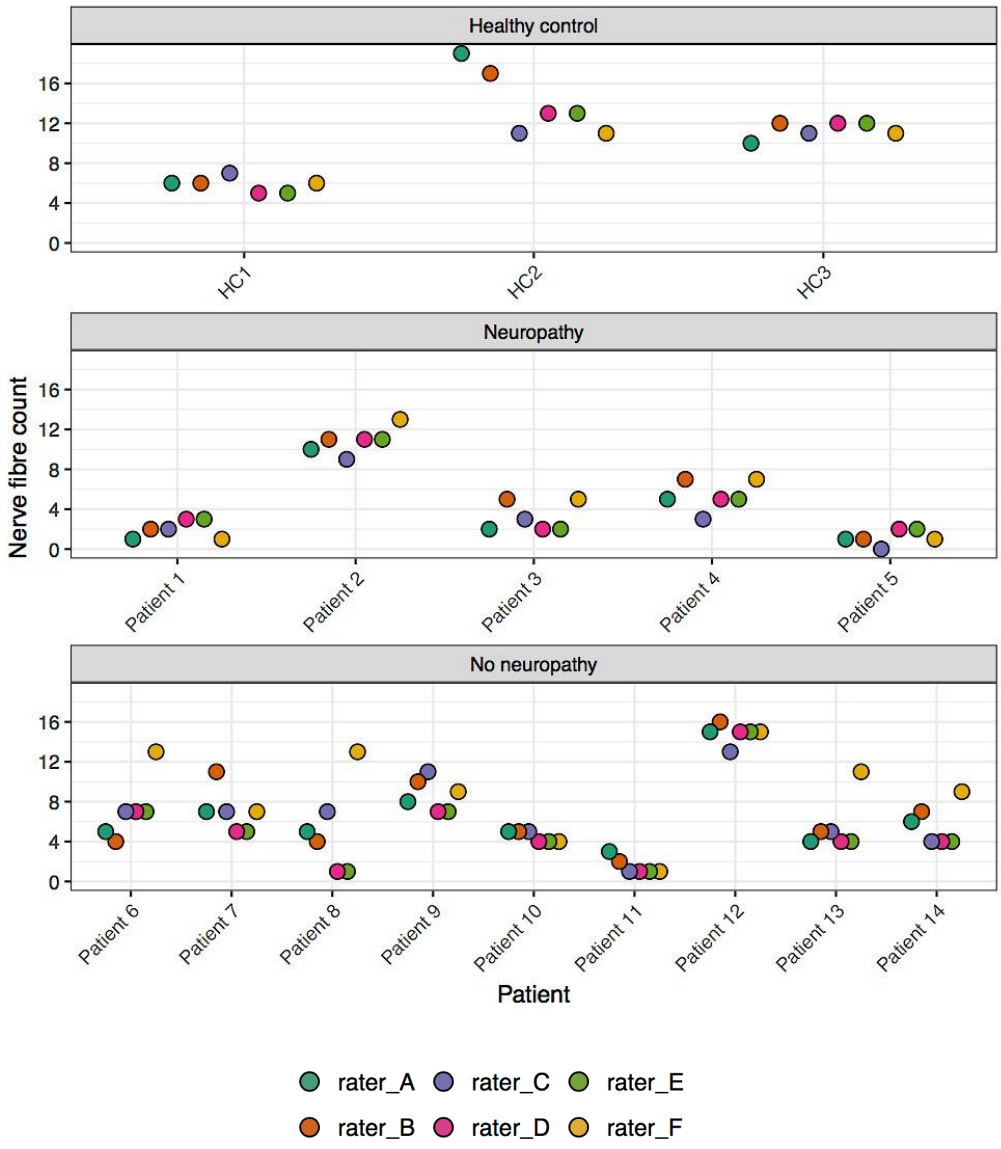

Figure 1. Mean intraepidermal nerve fibre count for each patient as determined by each rater

Appendix 5

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Polymorphisms in *IL10* may alter CD4 T-cell counts in Indonesian HIV patients beginning antiretroviral therapy

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ABSTRACT

Interleukin 10 (IL-10) is a potent anti-inflammatory cytokine influenced by single nucleotide polymorphisms (SNP) located in upstream regulatory regions. Here we address the effects of five SNP (rs1518111, rs3021094, rs3024491, rs1800872 and rs1800871) on CD4 T-cell counts in Indonesian HIV patients assessed before ART and over 12 months on treatment. Heterozygosity at rs1518111 or rs1800872 associated with low CD4 T-cell counts at all time points. Both alleles were carried in two haplotypes. Haplotype **2**1122 (present in 30% of participants) associated with low CD4 T-cell counts, whereas **2**122**2** (in 6% of participants) did not. Hence untyped SNP(s) tagged by **2**1122 may depress CD4 T-cell counts. The association with heterozygosity suggests synergy with an allele from a haplotype lacking rs1518111 and/or rs1800872.
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1. Introduction

CD4 T-cell counts are a reliable prognostic marker of HIV disease progression and risk of opportunistic infections. HIV-infected individuals differ in their rate of disease progression and their response to antiretroviral therapy (ART). This suggests complex interactions between the virus, the environment and the host genome. Host genes known to affect HIV disease include those encoding chemokine receptors, human leukocyte antigens (HLA), cytokines and apoptosis-related genes [1,2].

Interleukin-10 (IL-10) is an anti-inflammatory and immunomodulatory cytokine produced by macrophages, monocytes, T-helper cells and B-lymphocytes. IL-10 can inhibit the production of pro-inflammatory cytokines and down-regulate the expression of HLA class I and II molecules. IL-10 production in humans is influenced by genetic factors, which consequently have potential to affect HIV disease. Previous studies have focused on single nucleotide polymorphisms (SNP) within the *IL10* promoter region, notably −1082G/A (rs1800896), −592C/A (rs1800872) and −819C/T (rs1800871), and generate conflicting results. For example; the A allele of rs1800872 and the "ATA" haplotype defined by the three SNP increased susceptibility to HIV infection and disease progression in Indian cohorts [3,4]. However in an African population, carriers of AA at rs1800872 had attenuated CD4 T-cell loss and a broader CD8 T-cell response to HIV peptides, whilst rs1800896 more clearly affected plasma IL-10 levels [5]. Similarly the A alleles were protective against HIV and Hepatitis B disease in an Estonian population using intravenous drugs [6]. Studies from the Indian sub-continent associate −1082G/A (rs1800896) and −592C/A (rs1800872) with susceptibility to tuberculosis amongst HIV patients, but results included associations with heterozygous carriage [7].

This highlights the need for further studies to identify *IL10* SNP that may affect CD4 T-cell counts before and on ART. A study of 21 SNP spanning *IL10* in Caucasians HIV patients confirmed the −1082G/A (rs1800896), −592C/A (rs1800872) and −819C/T (rs1800871) haplotype as the most informative marker of disease progression but showed that it tagged and its effects were modified by broader *IL10* haplotypes. Interestingly, no clear associations were found in African Americans [8].

The patterns may also be distinct in Asians, so we have investigated associations between five polymorphisms in *IL10*

Abbreviations: ART, antiretroviral therapy; IL, interleukin; SNP, single nucleotide polymorphism.

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Table 1
Two *IL10* SNPs associated with low CD4 T-cell counts before and on ART.

SNP ID	V0/V3 V6/V12[a]	CD4 T-cell counts[b]			P-value[c]		
		1,1	1,2	2,2	1,1 vs 1,2	1,1 vs 2,2	1,2 vs 2,2
rs1518111 C (0.40)[d]	V0	72(9–199) (n = 30)	48(3–191) (n = 30)	74(2–187) (n = 15)	**0.02**	0.18	0.91
	V3	212(79–601) (n = 28)	169(7–492) (n = 25)	148(11–410) (n = 14)	*0.08*	**0.04**	0.50
	V6	226(118–516) (n = 26)	173(6–501) (n = 23)	221(20–394) (n = 13)	**0.04**	0.31	0.83
	V12	304(171–763) (n = 24)	242(121–616) (n = 21)	328(44–736) (n = 12)	*0.057*	0.72	0.67
rs3021094[e] G (0.46)	V0	49(2–187) (n = 24)	65(3–198) (n = 26)	69(9–199) (n = 19)	0.72	0.12	0.26
	V12	283(44–736) (n = 18)	285(121–603) (n = 20)	304(171–763) (n = 16)	0.87	0.5	0.32
rs3024491[f] A (0.06)	V0	62(2–199) (n = 68)	94(4–191) (n = 8)	–	0.49	–	–
	V12	282(44–763) (n = 53)	377(101–496) (n = 6)	–	0.38	–	–
rs1800872 G (0.42)	V0	69(9–199) (n = 29)	45(3–191) (n = 32)	52(2–187) (n = 16)	**0.03**	0.1	0.83
	V3	206(79–601) (n = 27)	136(7–492) (n = 27)	159(11–410) (n = 15)	**0.03**	0.08	0.80
	V6	242(118–516) (n = 25)	157(6–501) (n = 25)	226(20–394) (n = 14)	**0.02**	0.45	0.39
	V12	304(171–763) (n = 23)	207(121–616) (n = 23)	298(44–736) (n = 13)	**0.01**	0.67	0.41
rs1800871 G (0.43)	V0	67(2–187) (n = 16)	48(3–198) (n = 29)	67(9–196) (n = 26)	0.21	0.26	0.82
	V12	286(144–763) (n = 22)	233(121–616) (n = 22)	328(44–736) (n = 12)	0.21	0.96	0.79

[a] V0: CD4 T-cell counts before ART. V3, V6 and V12: CD4 T-cell counts after 3, 6 and 12 months on ART.
[b] Median (range) CD4 T-cell counts (cells/ul).
[c] Non-parametric Mann-Whitney test analysing association between the *IL10* genotypes and CD4 T-cell counts.
[d] Minor allele (MAF), where MAF are calculated from samples genotyped successfully.
[e] Observed allele frequencies deviated from the expectations of HWE ((X^2 4.01 > X^2 0.05, 1 *df* (3.84)).
[f] (1,2) and (2,2) were merged as <5 individuals carried the (2,2) genotype.

Table 2
One haplotype associated with low CD4 T-cell counts before and on ART.

Haplotype Sequence[a]	V0/V12	CD4 T-cell counts[c]			P-value[d]		
		0	1	2	1 vs 2	0 vs 2	0 vs 1
12111 (0.41)[b]	V0	50(2–198) (n = 31)	62(3–196) (n = 27)	69(9–199) (n = 21)	0.17	0.12	0.81
	V12	253(44–736) (n = 24)	289(121–567) (n = 20)	304(171–763) (n = 17)	0.46	0.21	0.75
21122 (0.3)	V0	72(7–199) (n = 38)	48(3–180) (n = 30)	50(2–166) (n = 11)	0.72	*0.07*	**0.02**
	V12	310(144–763) (n = 29)	207(101–616) (n = 23)	285(44–736) (n = 9)	0.48	0.61	**0.003**
11111 (0.14)	V0	63(2–199) (n = 62)	55(8–198) (n = 17)	–	–	–	0.61
	V12	288(44–763) (n = 49)	276(141–616) (n = 12)	–	–	–	1.0
21222 (0.06)	V0	55(2–199) (n = 71)	94(4–191) (n = 8)	–	–	–	0.44
	V12	282(44–763) (n = 55)	377(101–496) (n = 6)	–	–	–	0.35
12112 (0.3)	V0	55(2–199) (n = 75)	144(21–198) (n = 4)	–	–	–	0.17
	V12	285(44–763) (n = 58)	441(144–603) (n = 3)	–	–	–	0.50

[a] Defined by SNP alleles in the order shown in Table 1.
[b] Haplotype frequencies determined using the most probable assignment for each individual.
[c] Median (range) CD4 T-cells/ul. 0, 1 and 2 represent patients without the haplotype or with 1 or 2 copies.
[d] Non-parametric Mann-Whitney test.

(rs1518111, rs3021094, rs3024491, rs1800872 and rs1800871) and CD4 T-cell count recovery in HIV-infected Indonesian patients beginning ART. We sought the haplotypes that best define the risk.

2. Materials and methods

2.1. Patients and routine assessments

A longitudinal study of 79 HIV-1 patients was conducted at the HIV clinic at Cipto Mangunkusumo Hospital in Jakarta, Indonesia. Patients were invited to participate if they began ART with <200 CD4 T-cells/uL between March 2013 and March 2014. They were tested serologically for HCV, for oral candidiasis by clinical examination and for pulmonary tuberculosis by chest X-ray and sputum acid bacilli smear. The study was approved by the Ethics Committee of Cipto Mangunkusumo Hospital and Universitas Indonesia. Written informed consent was obtained from individuals who agreed to participate. CD4 T-cells were quantified by standard flow cytometry from the commencement of triple therapy (V0) including lamivudine, zidovudine, nevirapine, stavudine, efavirens and/or tenofovir. Most individuals were re-tested at 3, 6 and 12 months (V3, V6, V12). Reasons for discontinuation included death from causes related to AIDS (5 patients), drug-induced allergy requiring discontinuation of ART and loss to follow up. Plasma HIV RNA loads were determined using COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Tests (version 2.0).

2.2. Genotyping

DNA was quantified using a Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA) and diluted to 50 ng/uL. *IL10* polymorphisms (rs1518111, rs3021094, rs3024491, rs1800872 and rs1800871) were genotyped using custom TaqMan OpenArray Genotyping Plates (Life Technologies, Grand Island, NY) [9]. DNA samples were diluted at 1:1 in TaqMan OpenArray Genotyping Master Mix for 50 cycles of PCR amplification. The data was analysed using the OpenArray SNP Genotyping Analysis software, and the genotypes were assigned manually. Chi Square analysis with 5% significance level and 1 degree of freedom was used to determine if the observed allele frequencies were in Hardy-Weinberg equilibrium (HWE). Haplotypes and their estimated frequencies were calculated using the fastPHASE algorithm with the default parameters and sampled from the observed genotypes 10,000 times [10]. Haplotypes with an estimated frequency <1% were excluded.

2.3. Statistical analyses

Statistical analyses were done with GraphPad Prism software (v6; Tree Star, La Jolla, CA), using non-parametric Mann-Whitney tests, Chi^2 or Fisher's exact tests. A 5% significance level ($P < 0.05$) was considered statistically significant.

3. Results and discussion

Seventy-nine Indonesian HIV-infected patients were screened for *IL10* polymorphisms and their effect on CD4 T-cell counts before ART and over 12 months on treatment. The cohort included 51(65%) males and had a median (range) age of 31(19–48) years. The CD4 T-cell count at baseline was 61(3–199) cells/ul. 37(47%) of the patients had pulmonary tuberculosis and 17(22%) were co-infected with Hepatitis C. Heterosexual transmission accounted for 46 subjects (58%), with homosexual transmission and intravenous drug use each accounting for 18%. This is a common pattern in many Asian centres.

No genotypes associated with pulmonary tuberculosis, candidiasis or HCV (data not shown). Table 1 summarises associations between the five *IL10* SNP genotypes and CD4 T-cell counts. As the *IL10* rs3024491 (2,2) genotype was rare, patients with this genotype were analysed with those carrying rs3024491 (1,2). Univariate analyses associated heterozygous carriage of the minor alleles of two SNP with CD4 T-cell counts recorded before ART (rs1518111; $p = 0.02$ and rs1800872; $p = 0.03$) and after 3, 6 and 12 months (see Table 1), so the effect remains evident on ART. rs1518111 and rs1800872 are in linkage disequilibrium (1000 Genomes, $D' = 1$, $R^2 = 0.925$) in East Asians and Europeans, so common associations are plausible.

The G allele of the commonly studied −1082G/A (rs1800896) was also checked and found to be rare in our Indonesian cohort (MAF = 0.09). Moreover it is not in linkage disequilibrium with rs1800872 in East Asians described in the 1000 genomes database. Accordingly, carriage of the minor (G) allele of rs1800896 did not affect CD4 T-cell counts before ART or after 12 months (data not shown, $p = 0.77–0.83$).

To resolve the effect of heterozygous carriage of rs1518111 and rs1800872 and address which SNP were responsible for the phenotype, we considered the haplotypes carried by individuals in the cohort. Fifteen haplotypes of the five *IL10* SNP described in Table 1 were derived. Five haplotypes occurred at an estimated frequency of 1% or greater and accounted for 94% of this population (Table 2). The alleles of the SNP in each haplotype are expressed as a 1 (major allele) or a 2 (minor allele), in chromosomal order as listed in Table 1. Carriage of the haplotype (**2** 1 1 **2** 2) was associated with low CD4 T-cell counts ($p = 0.02$, Table 2). This haplotype contains the minor alleles of three SNP, where the first (rs1518111) and fourth (rs1800872) were associated with CD4 T-cell counts (Table 1). These two minor alleles also occurred in a rarer haplotype (**2** 1 2 **2** 2) that showed no association with low CD4 T-cell counts. This suggests that neither SNP is directly responsible for the low CD4 T-cell counts – rather the effect appears to reside with an unknown SNP tagged by the (**2** 1 1 **2** 2) haplotype.

In conclusion; our data links heterozygosity at *IL10* rs1518111 and rs1800872 with low CD4 T-cell counts in untreated HIV-infected Indonesian patients and on ART. Derivation of 5-SNP haplotypes demonstrated that these SNP are not directly responsible for the effect on CD4 T-cell counts. Moreover the consistent association with heterozygosity suggests synergy between SNP carried in distinct haplotypes, perhaps including the distal promoter region [8]. As data in the 1000 Genomes database show distinct *IL10* haplotypes carried by different ethnic groups, studies of CD4 T-cell recovery in other ethnicities may reveal which SNP are critical. As IL-10 is critical for limiting inflammation and the host immune response to pathogens, the association with *IL10* haplotypes confirms that these pathways influence CD4 T-cell loss and recovery on ART.

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Appendix 6

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