AN IMMUNOHISTOCHEMICAL ASSESSMENT OF ENDOMYOCARDIAL BIOPSY SPECIMENS FROM THE SOUTH AFRICAN ARRHYTHMOGENIC RIGHT VENTRICULAR CARDIOMYOPATHY REGISTRY

MMed Anatomical Pathology

Division of Anatomical Pathology, University of Cape Town, Cape Town, South Africa

University of Cape Town

I declare that this research report is based on independent work. This work has not been submitted to another university for a degree and this work has not been published.

Nicole Morse

Supervisor:

Prof H.C. Wainwright

University of Cape Town

Anzio Road Observatory 7925

Cape Town South Africa

Email: Helen.Wainwright@uct.ac.za

Co-Supervisor:

Prof B Mayosi

University of Cape Town

Anzio Road Observatory 7925

Cape Town South Africa

Email: Bongani.Mayosi@uct.ac.za

The copyright of this thesis vests in the author. No quotation from it or information derived from it is to be published without full acknowledgement of the source. The thesis is to be used for private study or non-commercial research purposes only.

Published by the University of Cape Town (UCT) in terms of the non-exclusive license granted to UCT by the author.

University of Cape Town

AN IMMUNOHISTOCHEMICAL ASSESSMENT OF ENDOMYOCARDIAL BIOPSY SPECIMENS FROM THE SOUTH AFRICAN ARRHYTHMOGENIC RIGHT VENTRICULAR CARDIOMYOPATHY REGISTRY

I declare that this research report is based on independent work. This work has not been submitted to another university for a degree and this work has not been published.

Dr Nicole Joy Morse

MRSNIC013

AKNOWLEDGEMENTS

I would like to acknowledge Ms Nafeesa Allie in the immunohistochemical laboratory of NHLS, Groote Scuur Hospital for her technical expertise and effort in cutting and staining sections as part of this study.

LIST OF ABBREVIATIONS

ARVC / D	arrhythmogenic right ventricular cardiomyopathy / dysplasia
AD	autosomal dominant
BC	*B*-catenin
BSA	body surface area
Cx43	connexin 43
DCMO	dilated cardiomyopathy
DP	desmoplakin
DSC	desmocollin
DSG2	desmoglein 2
DSP	desmoplakin
ECG	electrocardiogram
EVG	elastin Von Gieson
EMB	endomyocardial biopsy
GSH	Groote Schuur Hospital
H&E	haematoxylin & eosin
HIV	human immunodeficiency virus
IHD	ischaemic heart disease
JUP	plakoglobin
LV	left ventricular
NHLS	National Health Laboratory Service
PG	plakoglobin
PSR	picro-sirius red
PKP2	plakophilin 2
PLAX	parasternal long-axis
PP	plakophilin
PSAX	parasternal short-axis
RV	right ventricle

RVOT	right ventricular outflow tract
SG	desmogleins
SCD	sudden cardiac death

PART A: PROTOCOL

PART B: LITERATURE REVIEW

PART C: MANUSCRIPT

PART D: SUPPORTING DOCUMENTATION

PART A: PROTOCOL

RESEARCH PROPOSAL

Title

An immunohistochemical classification of endomyocardial biopsy specimens from the South African Arrhythmogenic Right Ventricular Cardiomyopathy Registry

Investigator

Dr Nicole Morse

MRSNIC013

Registrar

Division of Anatomical Pathology

Introduction

Arrhythmogenic right ventricular cardiomyopathy / dysplasia (ARVC/D) is a genetic disease causing fibro-fatty replacement of the right ventricular myocardium, resulting in cardiac arrhythmias and sudden death. Part of the diagnostic work up for these patients includes a biopsy of the endocardium which has historically been difficult to interpret and of limited value in the early stages of disease. This study will focus on novel immunohistochemical stains of the cardiac desmosomes. These will be used to try to aid in the early diagnosis of ARVC.

Supervisors

Professor H Wainwright, Principle specialist, Division of anatomical pathology University of Cape Town and Groote Schuur Hospital.

Professor B Mayosi, Head Department of Medicine, University of Cape Town and Groote Schuur Hospital. Principle investigator: Arrhythmogenic Right Ventricular Cardiomyopathy (ARVC) registry of South Africa.

Aims and objectives

Aim:

To classify the biopsy specimens of the ARVC registry into diagnostic, suspicious and non-diagnostic categories

Objective:

1: To ascertain if immunohistochemistry of cardiac desmosomes on biopsies from a septal location are of diagnostic value.

2: If immunohistochemistry is successful on cardiac biopsies to determine if there is a role for immunohistochemistry on skin biopsies.

Background

Arrhythmogenic right ventricular dysplasia/cardiomyopathy (ARVD/C) is a genetic disease of autosomal dominant and occasionally recessive inheritance with variability in its penetrance and expression[1] . It has been defined by the World Health Organisation/ International Society and Federation of Cardiology Task Force as a myocardial disease "characterised by fibro-fatty replacement the right ventricle (RV) myocardium, initially with typical regional and later global RV and some left ventricular involvement". It is associated with ventricular dilatation, arrhythmias, ECG abnormalities and sudden cardiac death.

Mutations have been described in five genes coding for the cardiac desmosome: Plakoglobin, desmoplakin, plakophilin 2 and desmoglein 2. Plakoglobin and desmoplakin mutations code for the autosomal recessive Naxos disease[1].

It has recently been reported that regardless of the genetic mutation, plakoglobin is the protein which fails to localise at the intercalated disc [1, 2].

Endomyocardial biopsy (EMB) showing fibrofatty replacement of the RV is recognised as a major criteria in the diagnosis of ARVD[3]. EMB is of value in excluding other causes of cardiomyopathy and arrhythmias such as amyloidosis, haemochromatosis and myocarditis[4].

Histomorphological parameters for ARVC have been defined as follows: residual myocytes less than 45%, fibrous tissue more than 40%, fatty tissue more than 3%. However it is recognized that that there will be a difference in these percentages depending on the site of the right ventricle biopsied as well as the distribution of the disease [4].

As ARVD is a segmental disease often only one of the biopsy samples will be diagnostic [5].

The right ventricular septum would be a preferred site of biopsy to avoid the complication of perforation, following biopsy from the thin RV free wall[4]. The septum has traditionally not been a recommended site for biopsy due to a low incidence of fibrosis and fatty infiltration [4]. However results of immunofluorescence stains have shown significant changes at this site in the absence of morphologic abnormalities [2].

Naxos disease is an autosomal recessive condition characterised by a triad of ARVD, wooly hair and plantopalmar keratoderma. It caused by a deletion of the plakoglobin gene on chromosome 17q21.

Plakoglobin is found in desmosomes within the epidermis [6]. It has been speculated that reduced expression of plakoglobin could be demonstrated by immunohistochemistry in skin biopsies from patients with ARVD. This could be of diagnostic significance if immunohistochemical stains prove the reduced expression as would obviate the need for an EMB [2].

The South African Arrhythmogenic Right Ventricular Cardiomyopathy Registry has been established under the Working Group of Registries of the Cardiac Arrhythmia Society of Southern Africa. The registry is based at the Cardiac Clinic, Groote Schuur Hospital, Cape Town.
Suspected cases from across the country are referred to the registry and if they meet the diagnostic criteria suspected patients are asked to partake in the registry. The registry has 6 branches: DNA and tissue bank; Epidemiology and family screening; Risk assessment and evaluation of therapy; Imaging; Pathology registry and tissue bank (EMB samples, explanted hearts) and Diagnostic validation [7].

Currently we have biopsies / explanted / post mortem hearts from 24 patients in the registry.

Methodology:

Procedures and protocols

Sample collection

We aim to collect ten cases of ARVC (diagnosed using clinical and radiologic characteristics as set out by the ARVC task force criteria), ten cases of HIV associated cardiomyopathy, ten cases of dilated cardiomyopathy (DCMO) and ten cases of transplanted donor hearts to use as normal controls. These will be retrieved from the retrieved from archives of the Division of Anatomical pathology at University of Cape Town.

Endomyocardial biopsy (EMB) sampling:

Biopsies already taken and registered with the South African Arrhythmogenic Right Ventricular Cardiomyopathy Registry will be retrieved from archives of the Division of Anatomical pathology at University of Cape Town.
For future biopsies the clinician will submit up to 4 biopsies in 10% formalin, each in separate container labeled with the anatomic site in the right ventricle.

Endomyocardial biopsy Processing:

Formalin fixed specimens will be placed in numbered cassettes and labeled "EMB".
Cassettes will be placed in tissue processor (Tissue Tec VIP 4) to be dehydrated by graded alcohols, washed and impregnated with paraffin.
The tissue is then orientated in wax blocks.

The blocks are cut on a microtome into 3-5*u*m slices.

Routine stains include Haematoxylin & Eosin (H&E), Elastin Von Gieson (EVG) / Picro-sirius red (PSR), Congo red, Perl's Prussian blue.

Slides to be issued to Dr HC Wainwright or Dr NJ Morse

For already processed tissues the H&E slides will be reviewed and PSR, Congo red and Perl's Prussian blue stains will be ordered if not already performed.

<u>Immunohistochemistry:</u>

Immunohistochemistry is used to detect antigens that will aid, confirm or exclude the diagnosis of a particular pathology or disease.

A primary antibody will bind to the targeted antigen.

A secondary antibody will bind to the primary antigen-antibody complex

In the presence of peroxidase and chromogen the secondary antibody turns brown at the site of the antigen.

The procedure used for immunohistochemistry is as follows:

1. Sections will be cut at 2-4 microns
2. Sections are floated onto APES coated slides and blotted
3. Heat fix the sections onto the slides by leaving slides on hot plate (60°C)
4. Dewax slides in xylol, rehydrate in graded alcohols.
5. Wash slides in running tap water
6. Perform appropriate antigen retrieval (citrate or EDTA depending on antibody used, see immunohistochemistry optimization section).
7. Block endogenous peroxidase activity by treating the slides with 1% hydrogen peroxide in methanol or distilled water for 5-10 minutes. Use a coplin jar.
8. Rinse in running tap water

9. Block for non-specific background staining by treating slides with normal goat serum (1:20) for 5-10 minutes
10. Drain the normal serum from the slides
11. Apply appropriately diluted primary antibody for 45 minutes. (See immunohistochemistry optimizing section on dilution values for each antibody.)
12. Rinse thoroughly with phosphate buffered saline (pH 7.6)
13. Apply Envision reagent, goat anti-rabbit antibody (secondary antibody), for 25-30 minutes
14. Repeat step 12
15. Apply chromogenic substrate (1ml buffer and 1 drop diaminobenzidene) for 3-5 minutes
16. Repeat step 12
17. Immerse slides in 1% copper sulphate for 3-5 minutes to enhance the diaminobenzidene
18. Rinse in running tap water
19. Counterstain in haematoxylin
20. Repeat step 12
21. Rinse the slides in Scotts tap water
22. Repeat step 12
23. Dehydrate and mount in entellan

Sections of the biopsy will be cut for immunohistochemical staining.

Stains used will be plakoglobin, Connexin 43, N-cadherin.

<u>Plakoglobin (gamma-catenin):</u>

Is a protein linking adhesion molecule present at the intercalated disc.

Reduced expression of plakoglobin has been shown in patients with ARVD [2].

This reduced signal is not only found in the RV free wall but also the septum and left ventricle. Reduced expression of plakoglobin appears to be the final common pathway in pathogenesis of ARVD, independent of which primary protein is affected [1]

The antibody can be purchased from Southern Cross Biotechnology.

Connexin 43:

Is a gap junction protein allowing direct gap junction intracellular communication and associated with growth regulation [8].

Shows reduced expression in all end stage heart failure specimens [2]

Antibody can be purchased from Laboratory Specialist Services.

N -cadherin:

N-cadherin is a non-desmosomal adhesion molecule and will be used as an internal marker of tissue quality. Decreased expression is seen as an indication of poor quality tissue on which a diagnosis cannot be made. [2]

N-cadherin can be purchased from Biocom Biotec.

Optimising the antibodies:

Due to the limited tissue available in EMB samples initial attempts at optimizing the antibodies were done on tissue from explanted hearts or postmortem tissues.

In the first run myocardium from a term, fresh still birth was used as normal control tissue.

ARVD =Arrhythmogenic right ventricular dysplasia

IHD = Ischaemic heart disease

1st run: N-Cadherin

Tissue	Antibody dilution	Buffer	Incubation time	Incubation temperature	Result
ARVD 1141/08	1:100	EDTA	45 min	Room temp	Very strong staining Background over stained
IHD 8872/08	1:100	EDTA	45 min	Room temp	Very strong staining Background over stained
Control (baby) PM74/09	1:100	EDTA	45 min	Room temp	Very strong staining Background over stained

1st run: Connexin 43

Tissue	Antibody dilution	Buffer	Incubation time	Incubation temperature	Result
ARVD 1141/08	1:200	EDTA	45 min	Room temp	Strong staining (aberrant)
IHD 8872/08	1:200	EDTA	45 min	Room temp	Strong staining (aberrant)
Control (baby) PM74/09	1:200	EDTA	45 min	Room temp	Weak staining (aberrant)

1st run: G-catenin

Tissue	Antibody dilution	Buffer	Incubation time	Incubation temperature	Result
ARVD 1141/08	1:100	EDTA	45 min	Room temp	Moderate staining (aberrant)
IHD 8872/08	1:100	EDTA	45 min	Room temp	Moderate staining (aberrant)
Control (baby) PM74/09	1:100	EDTA	45 min	Room temp	No staining (aberrant)

Due to the poor staining of Connexin 43 and G-catenin in the control tissue it was felt that adult heart should be used in the next run as a control.

The N-cadherin was diluted to1:200 in the second run to try remove the background staining.

New cases were selected for the ARVD and IHD as the both the Connexin 43 and G-catenin were showing aberrant staining.

2nd run: N-Cadherin

Tissue	Antibody dilution	Buffer	Incubation time	Incubation temperature	Result
ARVD 13560/05	1:100	EDTA	45 min	Room temp	Good staining
IHD 23349/07	1:100	EDTA	45 min	Room temp	Weak staining
Control (adult)	1:100	EDTA	45 min	Room temp	Good staining

2nd run: Connexin 43

Tissue	Antibody dilution	Buffer	Incubation time	Incubation temperature	Result
ARVD 13560/05	1:200	EDTA	45 min	Room temp	No Staining
IHD 23349/07	1:200	EDTA	45 min	Room temp	No staining
Control (adult)	1:200	EDTA	45 min	Room temp	No staining

2nd run: G-catenin

Tissue	Antibody dilution	Buffer	Incubation time	Incubation temperature	Result
ARVD 13560/05	1:200	EDTA	45 min	Room temp	Good staining
IHD 23349/07	1:200	EDTA	45 min	Room temp	Weak staining
Control (adult)	1:200	EDTA	45 min	Room temp	Good staining

Persistent staining of G -catenin in ARVD tissue was not expected, however the supplementary appendix to the Asimaki et al article "A New Diagnostic Test for Arrhythmogenic Right Ventricular Cardiomyopathy" specifies the use of citrate buffer [2]. This will be used in the 3rd run.
The poor staining of Connexin 43 cannot be explained as only the tissue samples were changed between run 1 and 2.

For the 3rd run new tissue samples were chosen, this time from endomyocardial biopsies. The control tissue used in this run was an endomyocardial biopsy from a donor heart transplant taken 1 week after surgery. This tissue had no evidence of rejection or structural pathology.

Once the antibodies have been optimized that procedure will become the standardized protocol for all further immunohistochemistry performed.

Skin biopsy samples:

Samples already submitted will be retrieved from the archives of the division of Anatomical Pathology, University of Cape Town.

For future biopsies the following protocol is recommended:

4mm punch biopsy of skin from the palm submitted in 10% formalin.

4mm punch biopsy from the forearm submitted in 2.5 % glutaraldehyde for electron microscopy.

Skin biopsy processing:

Paraffin sections will be prepared as for the EMB specimens.

Routine staining will only be an H&E.

Specimen will be examined for morphologic abnormalities of the keratin and epidermis.

Skin biopsy immunohistochemistry:

If the N-cadherin and plakoglobin immunohistochemistry is statistically significant on the cardiac biopsies then the same antibodies will be applied to the skin biopsies.

After antibody optimisation 10 skin biopsies from genetically or clinically proven ARVD patients and 10 skin biopsies from normal patients will be retrieved from archives.

The immunohistochemical panel of N-cadherin and G-catenin will be applied to the samples. With the pathologists blinded to the case the slides will be reviewed by Dr HC Wainwright and Dr NJ Morse. The results will be compared to both the EMB findings and clinical diagnosis.

Envisaged outputs/outcomes

Endomyocardial biopsy immunohistochemistry:

Decreased plakoglobin expression is expected in ARVD patients' versus normal controls with normal N-cadherin staining in both groups.

Skin immunohistochemistry:

Decreased plakoglobin expression is expected in ARVD patients' versus normal controls with normal N-cadherin staining in both groups.

Impact

If the results of the immunohistochemistry are statistically significant then the antibodies will be used as a diagnostic investigation in our routine practice for future EMB specimens.

If the antibodies are shown to be of use in EMB specimens then further research can done to see if staining of skin biopsies could be used in the diagnosis of ARVD rather than the invasive procedure of an EMB. This would be a great cost saving strategy as well as reducing the morbidity associated with endomyocardial biopsies.

This project falls under the collaborative Arrhythmogenic Right Ventricular Cardiomyopathy (ARVC) registry of South Africa, a multicentre study established under the Working Group of Registries of the Cardiac Arrhythmia Society of Southern Africa.

It will be used for a MMed (Anat Path) degree for Dr N Morse.

Budget

Budget requested from the NHLS Research Trust

	Consumables	Cost - Rands
1	Connexin 43 antibody 100ul	3941
2	Connexin 43 antibody 100ul	3941
3	N - cadherin antibody 100ul	8946
4	N - cadherin antibody 100ul	8946
5	G - catenin antibody 1ml	4592
6	G - catenin antibody 1ml	4592
7	Goat anti-rabbit antibody	10876
8	Diaminobenzidene 110ml	1845
9	Goat serum 10ml	300
10	Phosphate buffered saline	1388
11	Entellan 500ml	264
12	Glass slides x 50	90
13	Glass slides x 50	90
14	Glass slides x 50	90
15	Glass slides x 50	90
16	Coverslips x 100	46
17	Coverslips x 100	46
18	Microtome blades x 50	818
19	Histological sections	12000
20	Disposable tubes x 1000	719
22	Pipette tips (100ul) x 900	300
23	Pipette tips (200ul) x 900	300

24	Pipette tips (1000ul) x 900	300
25	Paper towels x 24 rolls	91
26	Conference attendance	2500
27	Conferance travel	4000
28	Antibody freight charges (Connexin 43)	3000
29	Antibody freight charges (G-catenin)	3000
30	Antibody freight charges (N-cadherin)	3000

Total R 80 111.00

Budget justification

The budget submitted includes the costs of the primary antibodies, secondary antibodies and consumables (pipette tips, buffers, slides) all used in the immunohistochemistry process. A budget is also included for conference and travel costs, so that this work may be presented.

REFERENCE LIST:

1. Tsatsopolou A, et al. Arrythmogenic right ventricular dysplasia, a cell adhesion cardiomyopathy: Insights into disease pathogenesis from preliminary genotype-phenotype assessment. Heart, BMJ 2006;92:1720-1723.
2. Asimaki A, et al. A new diagnostic test for arrhythmogenic right ventricular cardiomyopathy. N Engl J Med 2009;360(11):1075-1084.
3. McKenna WJ. Diagnosis of arrythmogenic right ventricular dysplasia/cardiomyopathy. British Heart Journal 1994;71:215-218.
4. Angelini A, et al. Endomyocardial biopsy in arrhythmogenic right ventricular cardiomyopathy. Am Heart J 1996;132(1 Pt 1):203-206.
5. Markus F. Autopsy and endomyocardial biopsy findings. Arrythmogenic RV Cardiomyopathy/Dysplasia Recent Advances. In: Markus F (ed) Arrythmogenic RV Cardiomyopathy/Dysplasia Recent Advances. 1 ed. Springer-Verlag Itlia: Assago; 2007, p38
6. Haftek M, et al. Interkeratinocyte adherens junctions: immunocytochemical visualization of cell-cell junctional structures, distinct from desmosomes, in human epidermis. J Invest Dermatol 1996;106(3):498-504.
7. Hendricks N. The South African Arrythmogenic Right Ventricular Cardiomyopathy Registry: A brief review and a status report. SA Heart 2008;5:148-154.
8. Pernick N. Connexin 43, in pathologyoutlines.com. 2009.
9. Basso C. Ultrastructural evidence of intercalated disc remodelling in arrhythmogenic right ventricular cardiomyopathy: an electron microscopy investigation on endomyocardial biopsies. European heart journal 2006;27:1847-1854.
10. Fletcher A, et al. Spectrum of pathological changes in both ventricles of patients dying suddenly with arrhythmogenic right ventricular dysplasia. Relation of changes to age. Histopathology 2006;48(4):445-452.
11. Trochim WMK. The T Test. Research Methods Knowledge Base 2006.

Protocol Summary

Title

An immunohistochemical classification of endomyocardial biopsy specimens from the South African Arrhythmogenic Right Ventricular Cardiomyopathy Registry

Investigator

Dr Nicole Morse (Division of Anatomical pathology, UCT)

Introduction

Arrhythmogenic right ventricular cardiomyopathy / dysplasia (ARVC/D) is a genetic disease causing fibro-fatty replacement of the right ventricular myocardium, resulting in cardiac arrhythmias and sudden death. Part of the diagnostic work up for these patients includes a biopsy of the endocardium which has historically been difficult to interpret and of limited value in the early stages of disease. This study will focus on novel immunohistochemical stains of the cardiac desmosomes. These will be used to try to aid in the early diagnosis of ARVC.

Aim of the study

To classify the biopsy specimens of the ARVC registry into diagnostic, suspicious and non-diagnostic categories. This will be done using immunohistochemical techniques.

Materials

Tissue blocks from ARVC registry patients (endomyocardial biopsies), patients known with dilated cardiomyopathy (DCMO), human immunodeficiency virus (HIV) associated cardiomyopathy and heart transplant biopsies will be retrieved from the Division of Anatomical Pathology archives.

Methodology

Immunohistochemistry will be applied to and compared between 10 cases each of ARVC, DCMO, HIV associated cardiomyopathy and cardiac transplant biopsies.

Impact

If the results of the immunohistochemistry are statistically significant then the antibodies will be used as a diagnostic investigation in our routine practice for future EMB specimens.

If skin biopsies show a statistically significant decrease in plakoglobin staining, skin biopsies could be used in the diagnosis of ARVD rather than the invasive procedure of an EMB.

Results

It will be used for a MMed (Anat Path) degree for Dr N Morse

To be submitted in peer-reviewed journals

To be presented in part at various national conferences

PART B: LITERATURE REVIEW

AN IMMUNOHISTOCHEMICAL ASSESSMENT OF ENDOMYOCARDIAL BIOPSY SPECIMENS FROM THE SOUTH AFRICAN ARRHYTHMOGENIC RIGHT VENTRICULAR CARDIOMYOPATHY REGISTRY

A LITERATURE REVIEW

by

Nicole Morse

Division of Anatomical Pathology, University of Cape Town, Cape Town, South Africa

Supervisor:

Prof H.C. Wainwright

University of Cape Town

Anzio Road Observatory 7925

Cape Town South Africa

Email: Helen.Wainwright@uct.ac.za

Co-Supervisor:

Prof B Mayosi

University of Cape Town

Anzio Road Observatory 7925

Cape Town South Africa

Email: Bongani.Mayosi@uct.ac.za

TABLE OF CONTENTS

OBJECTIVES

SEARCH STRATEGY

QUALITY CRITERIA

INTRODUCTION

CHAPTER 1 CLINICAL

CHAPTER 2 GENETICS

CHAPTER 3 BIOPSY AND AUTOPSY FINDINGS

CHAPTER 4 TASK FORCE CRITERIA

CHAPTER 5 CELL ADHESION

CHAPTER 6 IMMUNOHISTOCHEMISTRY

CHAPTER 7 SKIN

SUMMARY

FURTHER RESEARCH

TABLES AND FIGURES

REFERENCES

TABLE OF CONTENTS……………………………………………….….1

OBJECTIVES…………………………………………………..……3

SEARCH STRATEGY……………………………………………..3

QUALITY CRITERIA………………………………………………3

INTRODUCTION………………………………………………...4

CHAPTER 1 CLINICAL ……………………………………………5

CHAPTER 2 GENETICS…………………………………………...6

CHAPTER 3 BIOPSY AND AUTOPSY FINDINGS……………………..7

CHAPTER 4 TASK FORCE CRITERIA……………………………11

CHAPTER 5 CELL ADHESION………………………………..……13

CHAPTER 6 IMMUNOHISTOCHEMISTRY…………………….…15

CHAPTER 7 SKIN………………………………………………17

SUMMARY……………………………………………………..…18

FURTHER RESEARCH…………………………………………..…19

TABLES AND FIGURES……………………………………………..19

REFERENCES……………………………………………..………25

OBJECTIVES

This literature review aims to assess the role of pathology, particularly routine histopathology and immunohistochemistry in the diagnosis of ARVC.

This will form part of a MMed minor dissertation in anatomical pathology.

SEARCH STRATEGY

A PubMed search using the words “arrhythmogenic right ventricular cardiomyopathy / dysplasia” was performed up until January 2014. Articles with genetic, histopathology and immunohistochemical content were focused on. Clinical and radiologic articles were excluded.

QUALITY CRITERIA

All forms of research were assessed, only case reports were excluded from the review.

INTRODUCTION

ARVC is defined as a disease of the heart muscle characterised, clinically, by non-ischaemic ventricular arrhythmias originating from the right heart muscle [1]. Pathologically it is defined by fibro-fatty replacement of the right ventricular myocytes [2]. Originally felt to be a developmental defect of the RV myocardium, similar to Uhl's anomaly, the disease was given the name "Arrhythmogenic right ventricular "dysplasia". Ongoing developments into the genetic and pathophysiology of the disease the concept evolved into it being accepted as a cardiomyopathy by the World health Organisation in 1997.[3] [4, 5]. However, further evidence suggesting significant involvement of both left and right ventricles has resulted in the term arrhythmogenic cardiomyopathy being used synonymously for ARVC [6].

The first historical reference to ARVC was in the eighteenth century when Giovanni Maria Lancisi, the physician of the pope, reported 4 generations of a family with right ventricular (RV) heart failure and sudden cardiac death (SCD) [7]. It was only in the early eighties that ARVC became recognised as a specific disease entity when a case series of 24 patients was published in Circulation [8] .

A genetic basis of transmission of the disease was then reported in 1988 [9]. They described an autosomal dominant transmission with a variable penetrance of approximately 50% of cases. An autosomal recessive form of the disease had already been described in 1986 [10]. This detailed a syndrome of palmoplantar keratoderma and cardiac failure within families from the Greek island of Naxos, from whence the name Naxos disease was coined [11].

CHAPTER 1

CLINICAL

Clinically patients present between the second and forth decades with palpitations, syncope, cardiac arrest and SCD. Males are more commonly affected than females (male: female ratio 3:1) [12, 13]. The reported incidences of symptoms vary between studies. Sudden cardiac death has been reported to be as high as 23%, palpitations vary from 27 – 67% and syncope from 26 -32% [14, 15]

Three patterns of the disease have been described: [16, 17]

1 - “Silent” phase with RV cardiomyopathic abnormalities in asymptomatic victims of SCD.

2 - “Overt” disease with RV (and possible left ventricular (LV)) structural changes resulting in symptomatic arrhythmias.

3 - “End-stage” biventricular cardiomyopathy resulting in progressive cardiac failure

ARVC is often mis-diagnosed (or not diagnosed) both clinically and at autopsy. Therefore an accurate estimate of prevalence is not easy to determine. A large series published on sudden deaths from the Veneto region of Italy showed a prevalence of 1:2000 but general prevalence is estimated to be 1:5000 [18].

CHAPTER 2

GENETICS

Up to 50 % of ARVC cases are autosomal dominant (AD) in transmission with variable expression and penetrance [19]. This incomplete penetrance and expression has resulted in a search for environmental factors (especially viruses) and other genetic modifiers (extradesmosomal genes) in addition to the known mutations.

Mutations have been identified in the five major components of the desmosome. These genes are: PKP2 – plakophilin 2; DSG2 – desmoglein 2; DSP – desmoplakin, DSC2 – desmocollin 2 and JUP – plakoglobin (PG) [20]. The majority of mutations are in the PKP2 gene. However, regardless of which desmosomal gene has the mutation there is always a decrease in plakoglobin protein at the intercalated disc. This decreased expression of plakoglobin has therefore become an area of interest as a possible diagnostic marker for ARVC [20, 21]. New mutations are being discovered for example transmembrane protein -43 (TMEM43) is found in families from Newfoundland. This mutation is highly lethal and fully penetrant with affected males more likely to develop heart failure early transforming growth factor β-3 (TGFβ3) modulates expression of genes encoding for cell–cell junction stability and over-expression may induce myocardial fibrosis by stimulating mesenchymal cells to proliferate and produce extracellular matrix components [79].

Autosomal recessive transmission is also associated with mutations in the plakoglobin and desmoplakin genes [22]. As would be expected in an AR condition the penetrance of the disease is almost 100%. The recessive cases always present with the classic hair and skin changes (woolly hair and palmoplantar keratoderma). The cardiac presentation is predominantly left sided involvement when one of the desmoplakin mutations is present [22].

CHAPTER 3

BIOPSY AND AUTOPSY FINDINGS

Fibro-fatty replacement of cardiac myocytes is the histologic hallmark of the disease. This predominantly, or at least initially, occurs in the RV [3, 9]. This process is transmural, starting in the epicardium and progressing towards the endocardium with sparing of the trabecular musculature [5, 8, 16, 23].

Grossly the heart is of normal weight for age and sex and can be completely unremarkable in the early phases of the disease. As the disease progressed the characteristic macroscopic findings can be appreciated. On external view the heart appears whitish/yellow over the RV were the fat has infiltrated. The RV is dilated and ventricular aneurysms are characteristic and may be multiple. The aneurysms are usually found in the “triangle of dysplasia”: the inferior, apical and infundibular areas [8]. Involvement of the LV is seen in about half of all cases and septal involvement in 20% of cases but this process is usually microscopic [3, 16, 23]. Only in end stage disease will biventricular involvement be seen grossly, usually in autopsy or explanted hearts [3, 24]. Mural thrombi can be found in aneurysms and organisation of thrombi results in thickening of the endocardium.

Two histologic variants have been described: A fatty / lipomatous variant and a fibro-fatty variant [3, 25]. The fatty variant is, however, always accompanied by a small amount of fibrous tissue and careful sampling is required to identify the fibrosis. This is important as fat alone is not diagnostic of ARVC, normal hearts have intramyocardial fat in the antero-lateral and apical areas of the RV. In these normal hearts the boundary between the inner myocardium and outer subepicardial fat is usually fairly distinct [26]. Adipositas cordis and cardiac lipomas are conditions associated with increased adipose tissue. Adipositas cordis usually occurs in obese patients with hypertension and coronary artery atherosclerosis. This

fat is usually subepicardial and pushes the myocytes apart rather than replacing them [26, 27].

An injury – repair mechanism is proposed as the mechanism responsible for the histologic findings [3, 28]. Myocyte death is accompanied by myophagocytolysis and fibrous tissue replaces the injured muscle. This is backed up by the findings of an associated chronic inflammatory infiltrate in 66 - 75% of cases [22, 23, 29]. Apoptosis is also suggested to play a role in the death of myocytes. Increased apoptotic bodies are found in patients with a new onset of symptoms [30, 31]. Apoptosis, necrosis and inflammatory infiltrates are common findings at autopsy as they are associated with a "hot phase" of the disease from which a patient is more likely to die. It is less common to find apoptotic bodies and necrosis in endomyocardial biopsy specimens and therefore these features are not needed to make a diagnosis of ARVC [30, 32].

Genetic investigations into the Wnt/B-cat signalling pathway have revealed how cardiac progenitor cells can by stimulated into adipocytic differentiation [33].

Due to the presence of accompanying inflammation it has been suggested that a viral cause is responsible for ARVC, especially in patients with no family history. Viral DNA has been detected within the myocardium [34] but there is criticism that these are secondary and settle in the degenerating myocardium rather than causing it [35].

The general consensus is that fat alone is not diagnostic of ARVC. Fat accompanied by replacement type fibrosis with or without degenerate myocytes is needed to make a histologic diagnosis.[22, 25, 26]

Various investigators have looked into which histologic findings are most sensitive and specific for the diagnosis of ARVC on an endomyocardial biopsy. To this end quantitative parameters have been researched to try and determine numerical valves of fibrosis and fat required to make the diagnosis of ARVC. In summary here are the various researchers' findings over the last 2 decades:

Angelini et al in 1993 and 1996 [36, 37] reported a 67% sensitivity and 92% specificity if any one of the following parameters was present: Residual myocytes <45%, fibrosis >40%, fat >3% of the cross sectional area of the biopsy.

Wichter et al in 1994 [38] used the criteria of either fatty or fibro-fatty infiltration accounting for > 25% of the biopsy surface as diagnostic of ARVC. This figure gave them a sensitivity of 66%

Turrini et al in 1999 [39] compared the biopsy results to clinical findings and reported fibrosis of >30% was a significant predictor of a decreased RV ejection fraction and characteristic ECG abnormalities. He did not demonstrate any association between fatty tissue and these clinical parameters. This association of fibrosis causing late potential's on ECG has also been demonstrated by both Mehta et al [40] and La Vecchia et al [41]

Chimenti et al in 2004 [29] showed mean values of myocytes 44%, fat 24% and fibrosis 26% can diagnosis ARVC cases from myocarditis and controls.

Basso et al in 2008 [32] simplified the morphometric values and found that the percentage of residual myocytes is the main diagnostic parameter. A value of < 64% is diagnostic of ARVC with a sensitivity of 60% and a specificity of 90%.

It is important to stress that these morphometric parameters are only of diagnostic value if taken from the right ventricular free wall, "triangle of dysplasia" and not from the right septum or left ventricle [22, 29, 32, 38]. Clinicians are often against biopsying the free wall as it is thin and liable to perforate with a resultant haemopericardium. However the risk of death from this procedure is estimated to be <0.2% [42]. ECG guidance of the biopsy has been shown to be a safe option with a major complication rate of <0.4% and no deaths reported in a series of 4301 procedures [43].

As far as histomorphometry can be used in aiding the diagnosis of ARVC one must always use this together with qualitative assessment of the cellular pathology. Features of myocyte

hypertrophy, cytoplasmic vacuolation and nuclear abnormalities are important histologic clues to the diagnosis [26, 32].

CHAPTER 4

TASK FORCE CRITERIA

The accepted gold standard for the diagnosis of ARVC is histological evidence of transmural fibro-fatty replacement of the RV myocardium. This is obviously only possible in the autopsy setting or on an explanted heart. Endomyocardial biopsy is not transmural and often taken from the region of the, uninvolved, septum [22]. This is not of much value in the clinical setting and in 1993 a task force was established to set out parameters on which to make a diagnosis of ARVC.

The task force established six areas were the evidence indicated a diagnosis of ARVC could be made. These included (I) Global/regional dysfunction and structural alterations; (II) Tissue characterisation of walls; (III) Repolarisation abnormalities; (IV) Depolarisation/conduction abnormalities; (V) Arrhythmias; (VI) Family history.

Major and minor criteria were established within the areas according to their specificity for the condition. The Task force proposed that the diagnosis of ARVC could be made by the presence of two major criteria or one major plus two minor criteria or four minor criteria.

These criteria required only fibrofatty replacement of the myocardium as a major criterion of the diagnosis. They did not require any quantitative values of fibrosis or fat. No minor criteria were included in “tissue categorisation of walls”. See table 1.

At the time it was acknowledged by the task force that this was a working framework and that as more genetic evidence was uncovered more definite criteria could be set out [19]. The 1994 criteria were modified to include first degree relatives with incomplete expression of the disease. This was done in order to try increase the sensitivity of diagnosis [44]. In 2010 new criteria were established to incorporate advances in technology and genetics [45].

The big change of the tissue characterisation group between 1994 and 2010 is that a quantitative not a qualitative value of fibrofatty replacement is now used. They have used the morphometric values proposed by Basso et al as diagnostic criteria [32]. The 2010 criteria state that residual myocytes <60% by morphometry (or <50% if estimated), with fibrous replacement of the RV myocardium is diagnostic. Fat is not required for the diagnosis. See table 2.

CHAPTER 5

CELL ADHESION

Cardiac myocytes have a unique structure which allows electrical impulses to pass from the end of one myocyte into the adjacent myocyte, this is called the intercalated disc. Within the intercalated disc are gap junctions, which allow electrical coupling of the myocytes by allowing free passage of ions across membranes of adjoining myocytes [46]. These gap junctions are vital for the coordinated muscle contraction of the heart [33]. Mechanical coupling is formed by two structures within the intercalated disc the desmosome and the adherens junction. Desmosomes are rigid structures and form a stable adhesion between myocytes [47, 48] (See figure 1). Adherens junctions are both mechanical adhesions and interact with PI3K to mediate downstream Akt signalling [48].

Gap junction remodelling occurs in most forms of heart disease, including dilated cardiomyopathies, ischaemic heart disease, hypertrophic conditions and ARVC [49]. Remodelling refers to fewer and smaller gap junctions within the intercalated disc; this is demonstrated on both electron microscopy and immunohistochemistry [50]. It is felt that the characteristic ECG abnormalities and arrhythmias of ARVC are due to gap junction remodelling resulting in conduction defects within the myocardium [51]. The major protein found in gap junctions is connexin 43 (Cx43)[52]

The electrical integrity of gap junctions is dependent on surrounding mechanical support. As gap junctions are stiff protein structures, they are prone to rupture under shear forces. The adjacent adhesion junctions are thought to protect the gap junctions by providing mechanical support [53, 54].

Adhesion junctions are formed by cadherins (E-cadherin and N-cadherin) on opposite cell membranes hooking together to form a zipper-like structure. The cadherins tail in the cytoplasm binds either plakoglobin (PG, *y*-catenin, JUP) or *B*-catenin (BC). PG and BC in

turn link to the actin cytoskeleton via *a*-catenin [47] . Initially PG and BC were thought to be the same protein because they were so similar; however BC's role in Wnt signalling led to it being recognised as a separate entity [33].

PG is the only junctional component found in both desmosomes and adherens junctions. Within the desmosome PG links cadherins (desmoglein and desmocolin) to desmin (intermediate filament) via desmoplakin and plakophilin [33, 55]. Unlike in adherens junctions BC cannot replace the role of PG within the desmosome if PG is lost. In the adherens junction PG and BC can bind E-cadherin with equal affinity but in the desmosome PG binds desmoglein at a much higher affinity than BC [56, 57].

As already mentioned PG and BC are structurally very similar and both are also involved in intracellular signalling not just in cell-cell adhesion. When the desmosome is disrupted, it causes PG to leave the junction and enter the cardiac progenitor cell nucleus [58, 59]. When in the nucleus PG has been shown to suppress BC transcription and allows activation of adipogenic genes [60]. See figure 2.

The currently accepted hypothesis is that desmosomes are weakened by a mutated protein resulting in their disruption. The myocytes are no longer able to handle the haemodynamic shearing forces and become separated. These detached myocytes undergo apoptosis and are replaced by fibrosis under the influence of transforming growth factor beta (TGF*B*). Disruption of the desmosome with nuclear accumulation of PG causes the suppression of BC and causes the fatty replacement of the cardiac muscle. [62]

CHAPTER 6

IMMUNOHISTOCHEMISTRY

Connexin 43 (Cx43) is a transmembrane channel protein found in the gap junction. This protein is decreased at the gap junction in hearts diseased by many conditions including ischaemia, hypertrophy and cardiomyopathies [63]. Down regulation of Cx43 is associated with life threatening arrhythmias in these diseased hearts [64, 65]. Initial studies on patients with Naxos disease and Carvajal syndrome showed reduced expression of Cx43 at the gap junction is these conditions [50, 66] .

In their sentinel paper, published in the New England Journal of Medicine, Asimaki et al stained for Plakoglobin in heart biopsies from ARVC patients and compared them with biopsies from normal controls, hypertrophic, ischaemic and dilated cardiomyopathies [67]. They also stained for Cx43 and N-cadherin. N-cadherin is used as an internal control to ensure adequate tissue quality for antigen retrieval.

Their study showed a decrease (not complete absence) of PG in ARVC cases with no decrease in PG staining any of the other cases. This decreased PG staining was found in biopsies from any site in the heart, not just those from the “triangle of dysplasia”. They were correctly able to identify ARVC cases from other diseased hearts with a sensitivity of 91%, a specificity of 82% and a positive predictive value of 83%.

They stressed that the difference in PG staining was due to a decreased level of protein expression, not due to a complete absence of the protein. To be able to demonstrate this they had to dilute the PG antibody to 1:50 000 before any clear differences between the diseases could be demonstrated. Also it was the combination of a decreased PG with normal N-cadherin that was needed. If both antibodies were decreased the tissue was felt to be of poor quality and not suitable for diagnostic interpretation.

Asimaki et al used IF and confocal microscopy to establish a loss of PG expression, they did not use IHC. Traditionally immunofluorescence is used to identify the target protein but this is limited by the need for frozen material. Immunoperoxidase has been adapted to use on paraffin embedded, formalin fixed tissue as used in most laboratories [67]. The authors added a supplementary appendix to their article outlining the methodology needed to use immunoperoxidase rather than immunofluorescence.

Follow up studies have had variable results in reproducing the results published by Asimaki et al. One study, using immunoperoxidase correctly identified ARVC cases 70% of the time by showing a decreased PG signal [68]. This study highlighted the importance of uniform handling of specimens and that staining needs to be done under identical conditions in order to obtain reliable results. They conclude with the remark that caution must be used when interpreting PG at the gap junction and note there is no gold standard by which ARVC can be diagnosed. The authors highlighted the importance of the dilution of the PG antibody. In immunoperoxidase staining a dilution was used of 1:50 000 by Asimaki et al, published in their supplementary appendix, as well by Noorman et al [67, 68]. Munkholm et al needed to dilute their antibody to 1 : 300 000 to show reduced PG staining [69]

Reduced PG immunoperoxidase signalling was found to have a sensitivity of 85% and specificity of 57% in ARVC patients by Munkholm et al [69]. These values are lower than those reported by Asimaki et al. In this study normal PG staining was reported in ARVC patients, resulting in a higher rate of false negatives. These authors concluded that reduced PG signalling is a frequent but not mandatory finding in ARVC.

The specificity of a reduced PG signal has also been questioned. Reduced PG signalling was found in 43% of non ARVC cases by Munkholm et al [69]. The authors themselves and other experts do point out that these may be cases of concealed ARVC [68]. However, PG has been shown to be decreased in other histologically distinct diseases like sarcoidosis and giant cell myocarditis [70].

CHAPTER 7

SKIN

Patients with the AR form of ARVC, Naxos disease, have the cardiomyopathy phenotype as well as palmoplanter keratoderma [10, 11].

Keratoderma is the thickening of the stratum corneum of the skin and is either genetically inherited or acquired [71]. A mutation in PG in Naxos disease results in both the cardiomyopathy as well as the over-cornification and epidermal thickening of the palms and soles [72].

Epidermal desmosomes provide the main structural adhesion between keratinocytes and resist mechanical stresses. These desmosomes are formed by transmembrane cadherins, which bind to PG, which in turn binds to the intermediate filament network via desmoplakin [73].PG is responsible for the anchorage of intermediate filaments to desmosomes [74].

A study of PG knockout mice showed loss of membranous staining of PG in the keratinocytes with associated inappropriate desmosome assembly and cell detachment [75]. However a study by Ruiz et.al shows the PG null mice had significant loss of desmosomes in the heart but not the epidermis [76].

The clinical significance of PG mutations in human epidermis is demonstrated by Pigors et al. They describe a homozygous nonsense PG mutation resulting in s lethal congenital epidermolysis bullosa [77]. Deqiang et al used immunofluorescence to reveal disrupted desmosomes and adherens junctions in PG mutant epidermis. They were also able to demonstrate a compensational increase in β-catenin at PG mutant cell-cell junctions [78].

SUMMARY

The literature fails to show a single test as being the gold standard for the diagnosis of ARVC outside of the autopsy / explanted heart setting. The diagnosis of ARVC remains a combined clinical, radiologic, genetic and pathologic diagnosis as is set out in the revised Task Force Criteria.

FURTHER RESEARCH

Additional studies need to look into the robustness of immunohistochemistry in the diagnosis of ARVC in cardiac biopsies. This immunohistochemistry also needs to be explored for its role in skin biopsies in patients with Naxos disease and AD forms of ARVC.

TABELS AND FIGURES

Table 1: 1994 task force Criteria for diagnosis of right ventricular dysplasia

(Adapted from [19])

I - Global and/or regional dysfunction and structural alterations*	IV - Depolarisation/conduction abnormalities
MAJOR **Severe dilatation and reduction of right ventricular ejection fraction with no (or only mild) LV impairment** **Localised right ventricular aneurysms (akinetic or dyskinetic areas with diastolic bulging)** **Severe segmental dilatation of the right ventricle** **MINOR** **Mild global right ventricular dilatation and/or ejection fraction reduction with normal left ventricle** **Mild segmental dilatation of the right ventricle** **Regional right ventricular hypokinesia**	**MAJOR** **Epsilon waves or localised prolongation (> 1 10 ms) of the QRS complex in right precordial leads (V1-V3)** **MINOR** **Late potentials (signal averaged ECG)** **V - Arrhythmias** **MINOR** **Left bundle branch block type ventricular tachycardia (sustained and non-sustained) (ECG, Holter, exercise testing).** **Frequent ventricular extrasystoles (more than 1000/24 h) (Holter)**
II - Tissue characterisation of walls **MAJOR** **Fibrofatty replacement of myocardium on endomyocardial biopsy** **III - Repolarisation abnormalities** **MINOR** **Inverted T waves in right precordial leads (V2 and V3) (people aged more than 12 yr; in absence of right bundle branch block)**	**VI - Family history** **MAJOR** **Familial disease confirmed at necropsy or surgery** **MINOR** **Familial history of premature sudden death (<35 yr) due to suspected right ventricular dysplasia.** **Familial history (clinical diagnosis based on present criteria)**

*Detected by echocardiography, angiography, magnetic resonance imaging, or radionuclide scintigraphy. ECG, electrocardiogram; LV, left ventricle.

Table 2: 2010 Modified task force criteria
(adapted from [45])

I - Global or regional dysfunction and structural alterations*	**IV - Depolarization/conduction abnormalities**
MAJOR By 2D echo • Regional RV akinesia, dyskinesia, or aneurysm and 1 of the following (end diastole): ○ PLAX RVOT > 32 mm (corrected for body size [PLAX/BSA] > 19 mm/m2) ○ PSAX RVOT > 36 mm (corrected for body size [PSAX/BSA] > 21 mm/m2) ○ *or* fractional area change < 33% By MRI • Regional RV akinesia or dyskinesia or dyssynchronous RV contraction *and* 1 of the following: ○ Ratio of RV end-diastolic volume to BSA > 110 mL/m2 (male) or >100 mL/m2 (female) ○ *or* RV ejection fraction <40% By RV angiography • Regional RV akinesia, dyskinesia, or aneurysm MINOR By 2D echo • Regional RV akinesia or dyskinesia *and* 1 of the following (end diastole): ○ PLAX RVOT > 29 to <32 mm (corrected for body size [PLAX/BSA] > 16 to < 19 mm/m2) ○ PSAX RVOT > 32 to < 36 mm (corrected for body size [PSAX/BSA] > 18 to < 21 mm/m2) ○ *or* fractional area change > 33% to < 40% By MRI • Regional RV akinesia or dyskinesia or dyssynchronous RV contraction *and* 1 of the following:	MAJOR • Epsilon wave (reproducible low-amplitude signals between end of QRS complex to onset of the T wave) in the right precordial leads (V1 to V3) Minor • Late potentials by SAECG in >1 of 3 parameters in the absence of a QRS duration of > 110 ms on the standard ECG • Filtered QRS duration (fQRS) >114 ms • Duration of terminal QRS < 40 µV(low-amplitude signal duration) > 38 ms • Root-mean-square voltage of terminal 40 ms < 20 µV • Terminal activation duration of QRS > 55 ms measured from the nadir of the S wave to the end of the QRS, including R´, in V1, V2, or V3, in the absence of complete right bundle-branch block **V - Arrhythmias** MAJOR • Nonsustained or sustained ventricular tachycardia of left bundle-branch morphology with superior axis (negative or indeterminate QRS in leads II, III, and aVF and positive in lead aVL) MINOR • Nonsustained or sustained ventricular tachycardia of RV outflow configuration, left bundle-branch block morphology with

<table>
<tr>
<td>○ Ratio of RV end-diastolic volume to BSA > 100 to
< 110 mL/m2 (male) or > 90 to < 100 mL/m2 (female)
○ or RV ejection fraction >40% to < 45%

II - Tissue characterization of wall
MAJOR
• Residual myocytes < 60% by morphometric analysis (or < 50% if estimated), with fibrous replacement of the RV free wall myocardium in >1 sample, with or without fatty replacement of tissue on endomyocardial biopsy
MINOR
• Residual myocytes 60% to 75% by morphometric analysis (or 50% to 65% if estimated), with fibrous replacement of the RVfree wall myocardium in >1 sample, with or without fatty replacement of tissue on endomyocardial biopsy

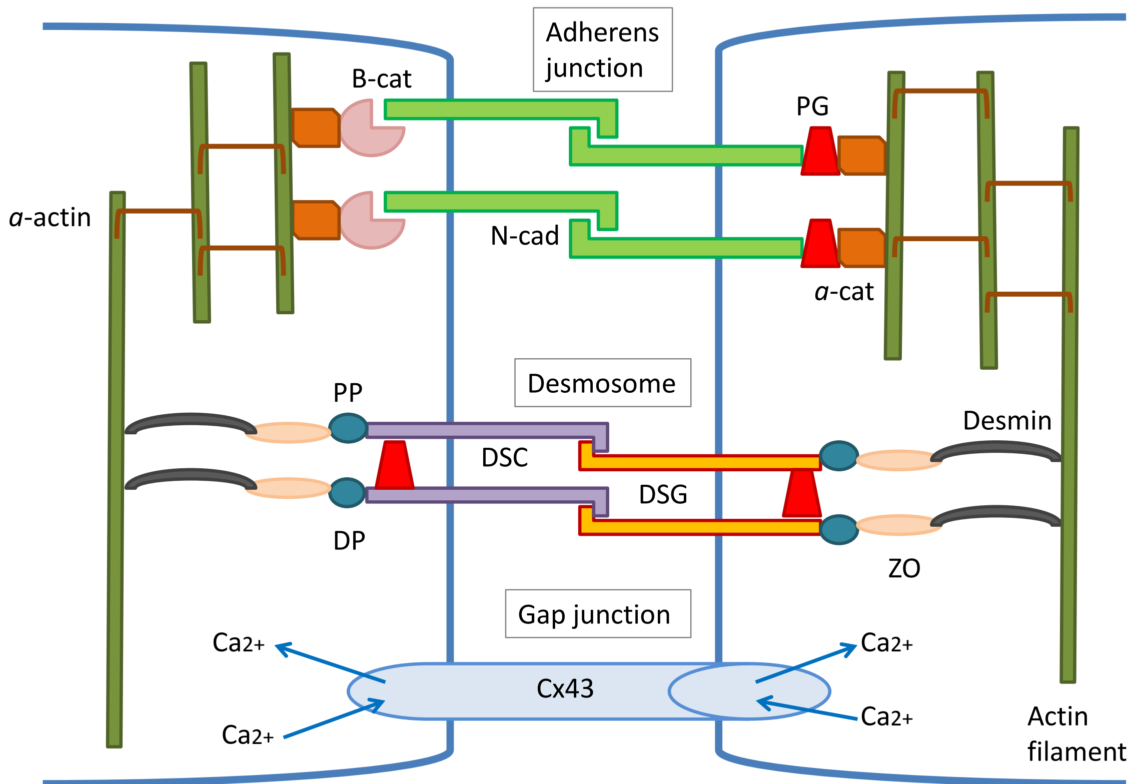
III - Repolarization abnormalities
MAJOR
• Inverted T waves in right precordial leads (V1, V2, and V3) or beyond in individuals 14 years of age (in the absence of complete right bundle-branch block QRS > 120 ms)
MINOR
• Inverted T waves in leads V1 and V2 in individuals 14 years of age (in the absence of complete right bundle-branch block) or in V4, V5, or V6
• Inverted T waves in leads V1, V2, V3, and V4 in individuals 14 years of age in the presence of complete right bundle-branch block</td>
<td>inferior axis (positive QRS in leads II, III, and aVF and negative in lead aVL) or of unknown axis
• .500 ventricular extrasystoles per 24 hours (Holter)

VI - Family history
MAJOR
• ARVC/D confirmed in a first-degree relative who meets current Task Force criteria
• ARVC/D confirmed pathologically at autopsy or surgery in a first-degree relative
• Identification of a pathogenic mutation† categorized as associated or probably associated with ARVC/D in the patient under evaluation
Minor
• ARVC/D confirmed pathologically or by current Task Force Criteria in second-degree relative
• History of ARVC/D in a first-degree relative in whom it is not possible or practical to determine whether the family member meets current Task Force criteria
• Premature sudden death (<35 years of age) due to suspected ARVC/D in a first-degree relative
• ARVC/D confirmed pathologically or by current Task Force Criteria in second-degree relative</td>
</tr>
</table>

Notes: Definite = 2 major OR 1 major + 2 minor; Borderline = 1 major + 1 minor OR 3 minor; Possible = 1 major OR 2 minor.

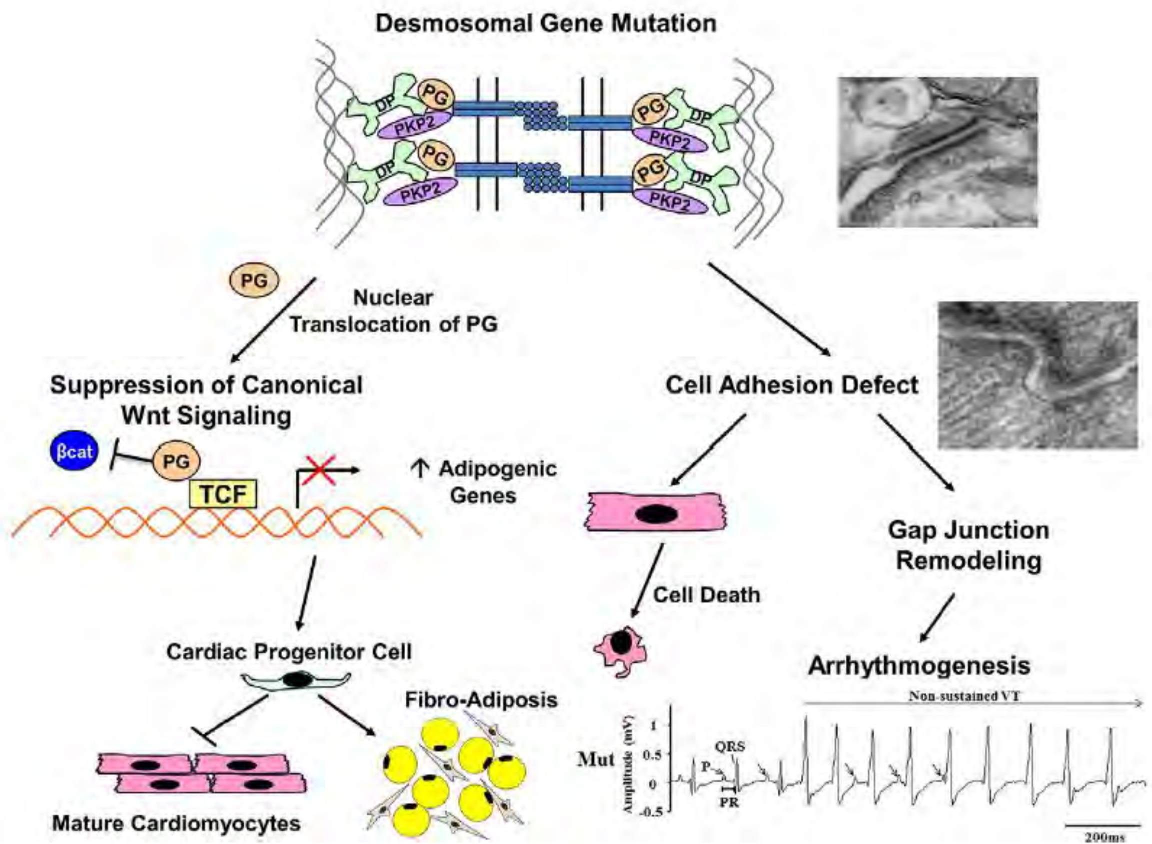
Abbreviations: PLAX, Parasternal Long-Axis; PSAX, Parasternal Short-Axis; RVOT, Right Ventricular Outflow Tract; BSA, body surface area.

Figure 1: Cell adhesion complexes (adapted from Guo D; J Mol Cell Cardiol 2011 [55])



Cell adhesion complexes in the heart illustrating the critical cell adhesion structures involved in cardiomyocyte–cardiomyocyte connection. Adherens junctions, desmosomes and gap junctions are shown. DSC=desmocollin; SG=desmogleins; PG=plakoglobin; DP=desmoplakin; PP=plakophilin;

Figure 2: Molecular pathogenesis of ARVC (Adapted from Lombardi R. Curr Opin Cardiol 2010; [61])



REFERENCES

1. Thiene G, Corrado D, Basso C. Arrhythmogenic right ventricular cardiomyopathy/dysplasia. Orphanet J Rare Dis 2007;2:45
2. Tabib A, et al. Circumstances of death and gross and microscopic observations in a series of 200 cases of sudden death associated with arrhythmogenic right ventricular cardiomyopathy and/or dysplasia. Circulation 2003;108:3000-3005.
3. Basso C, et al, Arrhythmogenic right ventricular cardiomyopathy. Dysplasia, dystrophy, or myocarditis? Circulation 1996;94(5):983-91.
4. Gerlis LM, Schmidt-Ott S, Ho SY, Anderson RH. Uhl's anomaly vs Arrhythmogenic right ventricular dysplasia. British heart journal 1993;69(2):142-150.
5. Richardson P, Bristow MW, et al. Report of the 1995 World health organisation/international society and federation of cardiology task force on the definition and classification of cardiomyopathies. Circulation 1996;93(5).
6. Tavora F. Distribution of biventricular disease in arrhythmogeniccardiomyopathy: an autopsy study. Human Pathology 2012;43(43).
7. Salvioni GM. De Motu Cordis et Aneurysmatibus Opus Posthumum In Duas Partes Divisum. 1728: Rome.
8. Marcus FI, et al. Right ventricular dysplasia: a report of 24 adult cases. Circulation 1982;65(2):384-398.
9. Nava A, et al. Familial occurrence of right ventricular dysplasia: a study involving nine families. J Am Coll Cardiol 1988;12(5):1222-1228.
10. Protonotarios N. Cardiac abnormalities in familial palmoplanter keratosis. British heart journal 1986;56:321-326.
11. Luderitz B. Naxos Disease. Journal Interventional cardiac electrophysiology 2003;9:405-406.
12. Basso C, Bauce B, Thiene G. Arrhythmogenic Right Ventricular Cardiomyopathy. Circ Arrhythm Electrophysiol 2012(5):1233-1246.

13. Corrado D, Pilichou K, Theine G, et al. Molecular biology and clinical management of arrhythmogenic right ventricular cardiomyopathy/dyspasia. Heart 2011;97:530-539.

14. Dalal D, Nasir K, Bomma C, et al. Arrhythmogenic right ventricular dysplasia: A United States experience. Circulation 2005;112:3823-32.

15. Nasir K, Bomma C, Thandri H, et al. Electrocardiographic features of arrhythmogenic right ventricular dysplasia/cardiomyopathy according to disease severity: a need to broaden diagnostic criteria. Circulation 2004;110(12):1527-1534.

16. Corrado D, Basso C, Theine G, et al. Spectrum of clinicopathologic manifestations of arrhythmogenic right ventricular cardiomyopathy/dysplasia: a multi center study. Journal American Coll Cardiology 1997;30:1512-1520.

17. Thiene G, Angelini A, Daliento L, Scognamiglio R, Corrado D., ed. Anatomoclinical aspects of arrhythmogenic right ventricular cardiomyopathy. Advances in cardiomyopathies., ed. C.F. Baroldi G, Goodwin JF, eds. 1990, Springer Verlag: Milano. 397-408.

18. Norman MW, McKenna WJ. Arrhythmogenic right ventricular cardiomyopathy/dysplasia: perspectives on disease. Z Kardiol 1999;88:550-554.

19. McKenna, WJ. Diagnosis of arrythmogenic right ventricular dysplasia/cardiomyopathy. British heart journal 1994;71:215-218.

20. Awad MM, Calkins H, Judge DP. Mechanisms of disease: molecular genetics of arrhythmogenic right ventricular dysplasia/cardiomyopathy. Nat Clin Pract Cardiovasc Med 2008;5(5):258-267.

21. Swope D, Li J, Radice GL. Beyond cell adhesion: the role of armadillo proteins in the heart. Cell Signal 2013;25:93-100.

22. Markus F. Autopsy and endomyocardial findings. Arrythmogenic RV Cardiomyopathy/Dysplasia Recent Advances. In: Markus F (ed) Arrythmogenic RV Cardiomyopathy/Dysplasia Recent Advances. 1 ed. Springer-Verlag Itlia: Assago; 2007.

23. Thiene G, Basso C. Arrhythmogenic right ventricular cardiomyopathy: An update. Cardiovasc Pathol 2001;10(3):109-117.

24. Thiene G, et al. Novel heart diseases requiring transplantation. Adv Clin Path 1998;2(1):65-73.

25. Thiene G, et al. Right ventricular cardiomyopathy and sudden death in young people. N Engl J Med 1988;318(3):129-33.

26. Basso C, Thiene G. Adipositas cordis, fatty infiltration of the right ventricle, and arrhythmogenic right ventricular cardiomyopathy. Just a matter of fat? Cardiovasc Pathol 2005;14(1):37-41.

27. D'Amati G, et al. Arrhythmogenic right ventricular cardiomyopathy: clinicopathologic correlation based on a revised definition of pathologic patterns. Hum Pathol 2001;32(10):1078-86.

28. Thiene G, et al. Right ventricular cardiomyopathy: is there evidence of an inflammatory aetiology? Eur Heart J 1991;12 Suppl D:22-5.

29. Chimenti C, et al. Histologic findings in patients with clinical and instrumental diagnosis of sporadic arrhythmogenic right ventricular dysplasia. J Am Coll Cardiol 2004;43(12):2305-2313.

30. Valente M, et al. In vivo evidence of apoptosis in arrhythmogenic right ventricular cardiomyopathy. Am J Pathol 1998;152(2):479-84.

31. Nagata M, et al. Apoptotic cell death in arrhythmogenic right ventricular cardiomyopathy: a comparative study with idiopathic sustained ventricular tachycardia. Jpn Heart J 2000;41(6):733-41.

32. Basso C, et al. Quantitative assessment of endomyocardial biopsy in arrhythmogenic right ventricular cardiomyopathy/dysplasia: an in vitro validation of diagnostic criteria. Eur Heart J 2008;29(22):2760-2771.

33. Swope D, Li J, Radice GL. Beyond cell adhesion: the role of armadillo proteins in the heart. Cell Signal 2013;25:93-100.

34. Bowles NE, et al. The detection of cardiotropic viruses in the myocardium of patients with arrhythmogenic right ventricular dysplasia/cardiomyopathy. J Am Coll Cardiol 2002;39(5):892-895.

35. Calabrese F, et al. Arrhythmogenic right ventricular cardiomyopathy/dysplasia: is there a role for viruses? Cardiovasc Pathol 2006;15(1):11-17.

36. Angelini A, et al. Endomyocardial biopsy in right ventricular cardiomyopathy. Int J Cardiol 1993;40(3):273-282.

37. Angelini A., et al. Endomyocardial biopsy in arrhythmogenic right ventricular cardiomyopathy. Am Heart J 1996;132(1 Pt 1):203-206.

38. Wichter T, et al. Regional myocardial sympathetic dysinnervation in arrhythmogenic right ventricular cardiomyopathy. An analysis using 123I-meta-iodobenzylguanidine scintigraphy. Circulation 1994;89(2):667-683.

39. Turrini P, et al. Late potentials and ventricular arrhythmias in arrhythmogenic right ventricular cardiomyopathy. Am J Cardiol 1999;83(8):1214-1219.

40. Mehta D, et al. Significance of signal-averaged electrocardiography in relation to endomyocardial biopsy and ventricular stimulation studies in patients with ventricular tachycardia without clinically apparent heart disease. J Am Coll Cardiol 1989;14(2):372-9; discussion 380-1.

41. La Vecchia L, et al. Ventricular late potentials, interstitial fibrosis, and right ventricular function in patients with ventricular tachycardia and normal left ventricular function. Am J Cardiol 1998;81(6):790-792.

42. Cunningham KS, Veinot JP, Butany J. An approach to endomyocardial biopsy interpretation. J Clin Pathol 2006;59(2):121-129.

43. Drury JH, Labovitz AJ, Miller LW. Echocardiographic Guidance for Endomyocardial Biopsy. Echocardiography 1997;14(5):469-474.

44. Marcus F, et al. Arrhythmogenic right ventricular dysplasia/cardiomyopathy (ARVD/C): a multidisciplinary study: design and protocol. Circulation 2003;107(23):2975-2978.

45. Marcus F, et al. Diagnosis of arrhythmogenic right ventricular cardiomyopathy/dysplasia: proposed modification of the task force criteria. Circulation 2010;121(13):1533-1541.

46. Kumar V. The Heart. Robbins and Cotran Pathologic Basis of disease. In: Kumar V (ed) Robbins and Cotran Pathologic Basis of disease,7 ed. Elsevier Saunders; 2005.

47. Shapiro L, et al. Structural basis of cell-cell adhesion by cadherins. Nature 1995;374(6520):327-337.

48. Tran NL, et al. Signal transduction from N-cadherin increases Bcl-2. Regulation of the phosphatidylinositol 3-kinase/Akt pathway by homophilic adhesion and actin cytoskeletal organization. J Biol Chem 2002;277(36):32905-32914.

49. Saffitz JE, Hames KY, and Kanno S. Remodeling of gap junctions in ischemic and nonischemic forms of heart disease. J Membr Biol 2007;218(1-3):65-71.

50. Kaplan SR, et al. Remodeling of myocyte gap junctions in arrhythmogenic right ventricular cardiomyopathy due to a deletion in plakoglobin (Naxos disease). Heart Rhythm 2004;1(1):3-11.

51. Asimaki A, Saffitz JE. Gap junctions and arrhythmogenic cardiomyopathy. Heart Rhythm 2012;9(6):992-995.

52. Thiene G. Arrhythmogenic right ventricular cardiomyopathy/dysplasia. Orphanet journal of rare diseases 2007;2(45).

53. Saffitz JE. Dependence of electrical coupling on mechanical coupling in cardiac myocytes: insights gained from cardiomyopathies caused by defects in cell-cell connections. Ann N Y Acad Sci 2005;1047:336-44.

54. Li J, et al. N-cadherin haploinsufficiency affects cardiac gap junctions and arrhythmic susceptibility. J Mol Cell Cardiol 2008;44(3):597-606.

55. Guo D, et al. Uncoupling between enhanced excitation-contraction coupling and the response to heart disease: lessons from the PI3Kγ knockout murine model. J Mol Cell Cardiol 2011;50:606-612.

56. Choi HJ, et al. Interactions of plakoglobin and beta-catenin with desmosomal cadherins: basis of selective exclusion of alpha- and beta-catenin from desmosomes. J Biol Chem 2009;284(46):31776-31788.

57. Zhurinsky J, Shtutman M, Ben-Ze'ev A. Plakoglobin and beta-catenin: protein interactions, regulation and biological roles. J Cell Sci 2000;113(Pt 18):3127-3139.

58. Simcha I, et al. Differential nuclear translocation and transactivation potential of beta-catenin and plakoglobin. J Cell Biol 1998;141(6):1433-1448.

59. Asimaki A. BIN1: A new biomarker to track ARVC? Heart Rhythm 2012;9(6):968-969

60. Garcia-Gras E, et al. Suppression of canonical Wnt/beta-catenin signaling by nuclear plakoglobin recapitulates phenotype of arrhythmogenic right ventricular cardiomyopathy. J Clin Invest 2006;116(7):2012-2021.

61. Lombardi R, Marian AJ. Molecular genetics and pathogenesis of arrhythmogenic right ventricular cardiomyopathy: a disease of cardiac stem cells. Curr Opin Cardiol 2010;25(3):222-228

62. Li D, Liu Y, Maruyama M, Zhu W, Chen H, Zhang W, Reuter S, Lin S, Haneline LS, Field LJ, Chen P, Shou W. Restrictive loss of plakoglobin in cardiomyocytes leads to arrhythmogenic cardiomyopathy. Human Molecular Genetics 2011;20:4582-4596.

63. Peters NS, et al. Reduced content of connexin43 gap junctions in ventricular myocardium from hypertrophied and ischemic human hearts. Circulation 1993;88(3):864-875.

64. Gutstein DE, et al. Heterogeneous expression of Gap junction channels in the heart leads to conduction defects and ventricular dysfunction. Circulation 2001;104(10):1194-1199.

65. Wiegerinck RF, et al. Transmural dispersion of refractoriness and conduction velocity is associated with heterogeneously reduced connexin43 in a rabbit model of heart failure. Heart Rhythm 2008;5(8):1178-1185.

66. Kaplan SR, et al. Structural and molecular pathology of the heart in Carvajal syndrome. Cardiovasc Pathol 2004;13(1):26-32.

67. Asimaki A, et al. A new diagnostic test for arrhythmogenic right ventricular cardiomyopathy. N Engl J Med 2009;360(11):1075-1084.

68. Noorman, M., et al., Reduced plakoglobin immunoreactivity in arrhythmogenic cardiomyopathy: methodological considerations. Cardiovasc Pathol. 22(5): p. 314-8.

69. Munkholm J, et al. Usefulness of immunostaining for plakoglobin as a diagnostic marker of arrhythmogenic right ventricular cardiomyopathy. Am J Cardiol 2012;109:272-275

70. Asimaki A, et al. Altered desmosomal proteins in granulomatous myocarditis and potential pathogenic links to arrhythmogenic right ventricular cardiomyopathy. Circ Arrhythm Electrophysiol 2011; 4(5):743-752.

71. Itin PH, Fistarol SK. Palmoplantar keratodermas. Clin Dermatol 2005;23(1):15-22.

72. McKoy G, et al. Identification of a deletion in plakoglobin in arrhythmogenic right ventricular cardiomyopathy with palmoplantar keratoderma and woolly hair (Naxos disease). Lancet 2000;355(9221):2119-2124.

73. Jamora C, Fuchs E. Intercellular adhesion, signalling and the cytoskeleton. Nat Cell Biol 2002;4(4):E101-108.

74. Acehan D, et al. Plakoglobin is required for effective intermediate filament anchorage to desmosomes. J Invest Dermatol 2008;128(11):2665-2675.

75. Li D, Zhang W, Liu Y, Haneline LS, Shou W. Lack of Plakoglobin in Epidermis Leads to Keratoderma. Journal Of Biological Chemistry 2012;287(13):10435-10443.

76. Ruiz P. Targeted Mutation of Plakoglobin in Mice Reveals Essential Functions of Desmosomes in the Embryonic Heart. Journal of Cell Biology 1996;135(1):215-225

77. Pigors M. Lack of plakoglobin leads to lethal congenital epidermolysis bullosa: a novel clinico-genetic entity. Human Molecular Genetics 2011:1–9

78. Li D, Zhang W, Liu Y, Haneline LS, Shou W. Lack of Plakoglobin in Epidermis Leads to Keratoderma. Journal Of Biological Chemistry 2012;287(13):10435-10443.

79. Iyer R. Arrhythmogenic right ventricular cardiomyopathy/Dysplasia (ARVC/D). American Journal of Medical Genetics Part C: Seminars in Medical Genetics. Special Issue: Disorders of Left Ventricular Trabeculation/Compaction or Right Ventricular Wall Formation 2013;163(3):185–197

PART C: MANUSCRIPT

AN IMMUNOHISTOCHEMICAL ASSESSMENT OF ENDOMYOCARDIAL BIOPSY SPECIMENS FROM THE SOUTH AFRICAN ARRHYTHMOGENIC RIGHT VENTRICULAR CARDIOMYOPATHY REGISTRY

A MANUSCRIPT

by

Nicole Morse

Division of Anatomical Pathology, University of Cape Town, Cape Town, South Africa

Supervisor:

Prof H.C. Wainwright

University of Cape Town

Anzio Road Observatory 7925

Cape Town South Africa

Email: Helen.Wainwright@uct.ac.za

Co-Supervisor:

Prof B Mayosi

University of Cape Town

Anzio Road Observatory 7925

Cape Town South Africa

Email: Bongani.Mayosi@uct.ac.za

Is decreased immunohistochemical plakoglobin expression a diagnostic test for arrhythmogenic right ventricular cardiomyopathy?

Nicole Morse*, Helen Wainwright*, Bongani Mayosi^

*Division of Anatomical Pathology, University of Cape Town, Groote Schuur Hospital
^Department of Medicine, Cardiology, University of Cape Town, Groote Schuur Hospital

SUMMARY

The diagnosis of arrhythmogenic right ventricular cardiomyopathy is dependent on clinical as well as histologic parameters, as set out in the revised task force criteria. At an ultrastructural level arrhythmogenic right ventricular cardiomyopathy is a disease of the cardiac desmosome. Plakoglobin is a desmosomal protein which, regardless of which arrhythmogenic right ventricular cardiomyopathy mutation is present, shows reduced expression in patients with arrhythmogenic right ventricular cardiomyopathy. An immunohistochemical antibody has been developed against plakoglobin and recent publications have suggested that a decrease in immunohistochemical expression of plakoglobin is diagnostic for arrhythmogenic right ventricular cardiomyopathy. We stained cardiac biopsies from eleven cases of arrhythmogenic right ventricular cardiomyopathy, ten cases of human immunodeficiency virus associated cardiomyopathy, eight cases of dilated cardiomyopathy and eleven cases of transplanted donor hearts with plakoglobin and N-cadherin immunolabels. The results showed that plakoglobin is a very difficult stain to interpret with only moderate inter-observer agreement (0.45 Kappa score). At best the sensitivity of this test is 72.73% but the specificity is only 32%. This study highlights the difficulties encountered in using plakoglobin immunohistochemistry and does not show a role for the routine use of plakoglobin in the diagnosis of arrhythmogenic right ventricular cardiomyopathy.

1. INTRODUCTION

arrhythmogenic right ventricular cardiomyopathy (ARVC) is a disease of the heart muscle characterised by non-ischaemic ventricular arrhythmias originating from the right heart muscle [1]. Pathologically it is defined by fibro-fatty replacement of the right ventricular myocytes [2].

The diagnosis of ARVC is challenging both clinically and pathologically. Fibro-fatty replacement of cardiac myocytes is the histologic hallmark of the disease. The general consensus is that fat alone is not diagnostic of ARVC. Fat accompanied by replacement type fibrosis with or without degenerate myocytes is needed to make a histologic diagnosis [3-5]. This fibro-fatty replacement should also be transmural within the right ventricular (RV) myocardium, as seen in the autopsy setting or in an explanted heart [2, 4]. Endomyocardial biopsies are not transmural and are often taken from the region of the uninvolved septum [3]. This is not of much value in the clinical setting and in 1993 a task force was established to set out parameters on which to make a diagnosis of ARVC. The task force established six areas where the evidence indicated a diagnosis of ARVC could be made. These included (I) Global/regional dysfunction and structural alterations; (II) Tissue characterisation of walls; (III) Repolarisation abnormalities; (IV) Depolarisation/conduction abnormalities; (V) Arrhythmias; (VI) Family history [3, 13].

Cardiac myocytes have a unique structure, the intercalated disc, which allows electrical impulses to pass from the end of one myocyte into the adjacent myocyte. Within this intercalated disc are gap junctions, which are responsible for electrical coupling of the myocytes. These gap junctions allow free passage of ions across membranes of adjoining myocytes [6]. Gap junction remodelling occurs in most forms of heart disease, including dilated cardiomyopathies, ischaemic heart disease, hypertrophic conditions and ARVC [7]. The major protein found in gap junctions is connexin 43 (Cx43)[8]. The electrical integrity of gap junctions is dependent on surrounding mechanical support in the form of the adhesion

junction [9, 10]. These adhesion junctions are formed by cadherins (E-cadherin and N-cadherin) on opposite cell membranes hooking together to form a zipper-like structure. The cadherin's tail in the cytoplasm binds either plakoglobin (PG) or *B*-catenin (BC). PG is the only junctional component found in both desmosomes and adherens junctions. Within the desmosome PG links cadherins (desmoglein and desmocolin) to desmin (intermediate filament) via desmoplakin and plakophilin [11, 12]. See figure 1.

Up to 50 % of ARVC cases are autosomal dominant (AD) in transmission with variable expression and penetrance [13]. Mutations have been identified in the five major components of the desmosome. These genes are: PKP2 – plakophilin 2; DSG2 – desmoglein 2; DSP – desmoplakin, DSC2 – desmocollin 2 and JUP – plakoglobin (PG) [14]. The majority of mutations are in the PKP2 gene. However, regardless of which desmosomal gene has the mutation there is always a decrease in plakoglobin protein at the intercalated disc. This decreased expression of PG has therefore become an area of interest as a possible diagnostic marker for ARVC [14, 15].

An antibody to PG has been developed and is commercially available in both immunofluorescence and immunoperoxidase forms. It has been suggested that this can be used to diagnose ARVC in cardiac biopsy specimens [16]. Traditionally immunofluorescence is used to identify the target protein but this is limited by the need for frozen material. Immunoperoxidase has been adapted for use on paraffin embedded, formalin fixed tissue as used in most laboratories [16].

The immunohistochemical diagnosis of ARVC is based on identifying a decrease in the staining intensity of PG, as a marker of the decreased PG expression, at the intercalated disc [16]. This decrease in staining intensity can be compared to the N-cadherin staining, which is present at the intercalated disk, but not the desmosome (i.e. the N-cadherin acts as an internal control of tissue quality).

Although the sentinel paper showed excellent sensitivity and specificity, follow up studies have had variable success in reproducing these results [17, 18]. Others have demonstrated

a decreased staining intensity of PG in other histologically distinct diseases like sarcoidosis and giant cell myocarditis [19].

In this study the authors aimed to assess the sensitivity and specificity of PG immunohistochemistry on cardiac biopsies from 3 different diseases (ARVC, dilated cardiomyopathy (DCM) and HIV associated cardiomyopathy).

2. MATERIALS AND METHODS

2.1. Study group

Paraffin embedded cardiac biopsies were obtained from the National Health Laboratory service (NHLS)/Groote Schuur Hospital (GSH) archives. In total 11 cases of ARVC (These cases were identified by Professor Mayosi - principle investigator ARVC registry of South Africa. The diagnosis of ARVC was made using clinical and radiologic characteristics as set out by the ARVC task force criteria), 10 cases of HIV associated cardiomyopathy, 8 cases of dilated cardiomyopathy (DCMO) and 11 cases of transplanted donor hearts to use as normal controls, were collected.

2.2. Immunohistochemistry

To optimise the antibodies prior to use on the cardiac biopsies, tissue was obtained from autopsy and explanted hearts from patients with ARVC, dilated cardiomyopathy and patients dying from non-cardiac causes. The immunohistochemical stains used were N-cadherin (Sigma, UK; diluted 1:100, EDTA buffer), G-catenin (PG) (Sigma, UK; diluted 1:50 000 then 1: 60 000, citrate buffer) and Connexin 43 (Zymed, UK; diluted 1:100, EDTA buffer).

Although the antibodies used were identical to those used by Asimaki et al, as set out in their supplementary appendix, we could not exactly reproduce their findings on the autopsy/explanted samples. Despite all efforts with dilutions and different retrieval systems, it was not possible to eliminate non-specific background staining on the Cx43 slides. The authors failed to show a loss of Cx43 staining in the diseased hearts as compared to the control tissue. For this reason it was decided to not include Cx43 in the rest of the study. Although Asimaki et al were able to show a decrease in PG staining at 1:50 000 dilutions, this study only observed this difference at a dilution of 1: 60 000.

Each biopsy was then stained with N – Cadherin and PG. Using the NHLS immunohistochemical protocol sections were cut at 3 microns and floated onto aminopropyltriethoxysilane coated slides. The sections were heat fixed onto the slides by

leaving slides on hot plate (60°C). The slides were dewaxed in xylol and rehydrated in graded alcohols. Antigen retrieval was performed using citrate or EDTA depending on antibody used (see above). Endogenous peroxidase activity was blocked by treating the slides with 1% hydrogen peroxide in methanol for 5 minutes. Normal goat serum (1:20) was used to block for non-specific background staining by treating slides with normal goat serum (1:20) for 5 minutes. The appropriately diluted primary antibody was then applied for 45 minutes and rinsed thoroughly with phosphate buffered saline (pH 7.6). Envision reagent, goat anti-rabbit antibody (secondary antibody) was applied for 25 minutes and the slides were again washed with buffered saline. A chromogenic substrate (1ml buffer and 1 drop diaminobenzidene) was applied for 3 minutes and washed before slides were immersed in 1% copper sulphate for 3 minutes. Haematoxylin was used as a counter stain and rinsed with Scott's tap water.

2.3. Immunohistochemical assessment

All cases were assessed by two pathologists (N.J.M. and H.C.W.) who were blinded to the clinical diagnosis. Each stain (PG and N-cadherin) was scored with a semi-quantitative assessment of intensity using the following criteria: 0, negative – no typical staining seen, even at 40 x objective; 1, weak – typical staining seen but only convincing when using 40 x objective; 2, moderate – typical staining seen, convincing using 20 x objective; 3, strong – obvious, typical staining at 4 x objective. See figure 2 and 3. (A similar semi-quantitative scoring system has been used in assessing the BRAF V600E antibody in colorectal carcinoma [21]). Typical staining had to be seen as a linear stain at the gap junction, perpendicular to the long axis of the myocyte, to be considered positive.

The same cases were then assessed qualitatively by the same two pathologists, without using a quantitative scoring system. The N-cadherin staining intensity was compared to the PG staining intensity of the same case. If the PG was weaker in staining intensity than the N-cadherin (i.e. less PG protein expression than N-cadherin protein expression) a diagnosis of ARVC was made on that case.

Each case was given an immunohistochemical diagnosis of: a) ARVC, b) possible ARVC or c) not ARVC. This diagnosis was based on the scoring intensity difference between the N-cad and PG. In ARVC the PG is expected to be weaker than N-cad hence the N-cad score was subtracted from the PG score. For example an N-cad score of 3 subtracted from a PG score of 1 would result of a value of -2.

If the N-cad scored 0 the case was discarded as the internal control had failed. If the G-cat stained the same (value 0) or stronger (value +1 to +2) than the N-cad, the case was diagnosed as not ARVC, see example Figure 4. A value of -1 was considered possible ARVC and a value of -2 or -3 was diagnostic of ARVC, see example figure 5. See Table 1.

2.4. Statistical analysis

Interobserver variability was determined using Landis and Koch's measurement of observer agreement for categorical data [22]. Sensitivity and specificity were determined using a 95% confidence interval.

3. RESULTS

3.1. Using the semi-quantitative assessment

Using the semi-quantitative scoring method N.J.M. had a sensitivity of 38.46% [95% CI: 14.00% to 68.36%] and a specificity of 39.29% [95% CI: 21.53% to 59.42%]. H.C.W. had a sensitivity of 23.08% [95% CI: 5.31 % to 53.80 %] and a specificity of 64.29% [95% CI: 44.07% to 81.33%].

If the "possible ARVC" were excluded from the statistical analysis the specificity of the test increased but the sensitivity decreased. See Table 2.

There was only fair agreement between N.J.M and H.C.W (Kappa 0.085).

3.2. Using qualitative assessment

Interobserver agreement and sensitivity of the test both improved when the immunohistochemical stains were assessed in a qualitative manner. However the specificity of the test decreased. See Table 2.

N.J.M sensitivity was 72.73% [95% CI: 39.08% to 93.65%] and specificity was 32% [95% CI: 14.99% to 53.50%]. H.C.W sensitivity was 63.64% [95% CI: 30.88% to 88.85%] and specificity was 40% [95% CI: 21.16% to 61.32%]. The Cohen's kappa agreement was moderate (0.45).

In a similar way to where "Possible ARVC" cases were excluded for in the semi-quantitative analysis, borderline diagnostic cases can be excluded from the qualitative analysis: Cases which had discordant diagnosis between the pathologists represent those where there is diagnostic difficulty in interpreting the difference in staining. Where there was interpretation agreement between the two pathologists the authors were able to demonstrate a reduced signal of PG in 70% (7/10) of the ARVC cases. However, PG was also decreased in 60% (4/6) of DCMO cases; 60% (3/5) of HIV cases and 85% (5/6) of transplants.

Example of a false positive diagnosis is given as figure 6. Both pathologists qualitatively diagnosed this case as ARVC, based on the striking loss of PG staining. However, this represents a biopsy from an HIV cardiomyopathy.

An example of a false negative diagnosis is given as figure 7. Both pathologists qualitatively diagnosed this case as “Non- ARVC” as no difference in N-cad and PG staining was observed. This is in fact a biopsy from an ARVC patient.

4. DISCUSSION

This is not an easy immunohistochemical stain to evaluate, as evidenced by only fair interobserver agreement (kappa value of 0.085) when a semi-quantitative method is applied. Other immunohistochemical stains where semi-quantitative scores are given, for example Her2Neu in breast cancer, clear guidelines exist for these staining patterns and intensity [20]. No guidelines are available for pathologists in trying to interpret a loss of staining of PG versus N-cadherin and this makes it difficult to use this test in routine practice. This lack of standardization in scoring these cases resulted in both poor sensitivities (38.46 and 23.08 %) and specificities (64.29 and 73.33%). When the borderline or ambiguous cases labelled "Possible ARVC" (i.e. those showing only a scoring intensity difference of -1) were excluded from the statistical analysis then an increased specificity was noted (73.33 and 85.71%) but at the expense of the sensitivity (27.27 and 0%). Therefore, a strong staining intensity difference (-2 or -3) between PG and N-cadherin could be used to confirm a diagnosis of ARVC, but not exclude it as the sensitivity of the test is too low.

If using the test in a qualitative manner the Kappa score increased to 0.45 - moderate agreement between the observers. The qualitative assessment also allowed for an improved sensitivity (72.73 and 63.64%) but the specificity decreased (32 and 40%) when compared to the semi-quantitative evaluation. The decreased specificity is due to a decrease in staining intensity difference being reported in the non ARVC cases. This trend was accentuated when the cases with discrepant diagnosis between pathologists were excluded. Where the two pathologists agreed there was a loss of staining a diagnosis of ARVC was made correctly in 7/10 cases of cases but a decreased staining, and hence a diagnosis of ARVC, was also observed in 12/17 of the non ARVC cases.

Although a qualitative analysis allows increased numbers of cases of ARVC to be diagnosed, it loses the specificity of the semi-quantitative analysis.

These results failed to demonstrate a role for PG immunohistochemistry in the diagnosis of ARVC. This is possibly due to a variety of different factors.

Cases of concealed ARVC may have resulted in the poor specificity of the test. However, the cases of HIV associated cardiomyopathy have all been extensively worked up and have a known pathogenesis (as opposed to dilated CMO where there is clinical overlap) and the transplant biopsies represent tissue taken from normal hearts where the probability of concealed ARVC is remote.

This study used archival cases where there was no uniform handling of the biopsy material, which may explain the poor sensitivities and specificities that are reported. However, all the cases were stained in the same batch, so variations in the antibody concentration and antigen retrieval techniques are unlikely to be the cause of the poor outcomes. Also, in everyday practice it is unlikely that cardiac biopsies are going to be batched for the immunohistochemistry; each case will be stained and reported as it comes into the laboratory.

This is a difficult immunohistochemical stain to interpret and even two pathologists with extensive exposure to this stain at best only achieved a kappa agreement that was moderate. This is likely due to the small nature of the cardiac biopsies themselves, as opposed to staining done on explanted or autopsy material. Also, the specific staining site (along the gap junction) may not be seen if the myocytes are cut along the short axis.

The antibody dilution seems to be critical when using PG immunohistochemistry in cardiac diseases. A reduced PG signal was shown to have a specificity of 82% by the Asimaki et al, and only their ARVC cases showed a decreased staining with no decrease in PG staining in any of the other cases. They were correctly able to identify ARVC cases from other diseased hearts with a sensitivity of 91%. They stressed that the difference in PG staining was due to a decreased level of protein expression, not due to a complete absence of the protein. To be able to demonstrate this they had to dilute the PG antibody to 1:50 000 before any clear differences between the diseases could be demonstrated.

Personal communication by the author with Dr Asimaki revealed that many laboratories from around the world have tried to use immunohistochemistry and have reported “varying success”.

Follow up studies have also had variable success in reproducing the results published by Asimaki et al. The study by Noorman et al, using immunoperoxidase, correctly identified ARVC cases 70% of the time by showing a decreased PG signal [18]. This study highlighted the importance of uniform handling of specimens and that staining needs to be done under identical conditions in order to obtain reliable results. They conclude with the remark that caution must be used when interpreting PG at the gap junction and note that there is no gold standard by which ARVC can be diagnosed. The authors highlighted the importance of the dilution of the PG antibody. An immunoperoxidase staining dilution of 1 : 50 000 was used by Asimaki et al, published in their supplementary appendix, as well as by Noorman’s et al [16, 18]. However in the publication by Munkholm et al a dilution of 1 : 300 000 was needed to show reduced PG staining [17]

Reduced PG immunoperoxidase signalling was found to have a sensitivity of 85% and specificity of 57% in ARVC patients by Munkholm et al [17]. These values are lower than those reported by Asimaki et al. In this study normal PG staining was reported in ARVC patients, resulting in a higher rate of false negatives. These authors concluded that reduced PG signalling is a frequent but not mandatory finding in ARVC.

The specificity reported in Asimaki et al study is in contrast to that of Noorman et al, in which reduced PG signalling was found in 43% of non ARVC cases. The authors themselves and other experts point out that these may be cases of concealed ARVC [18]. PG has been shown to be decreased in other histologically distinct diseases like sarcoidosis and giant cell myocarditis [19] where there is no probability of underlying occult ARVC.

Suggestions to improve the interpretation of this PG immunohistochemical stain include the use of a red chromagen. The presence of paranuclear lipofuscin can be misinterpreted as PG. Perhaps, had a red chromagen been used, the stain would have been easier to

distinguish from lipofuscin. It also is likely that it is easier to use this antibody in an immunofluorescence setting where the interpretation is more obvious.

It is also possible that more consistent results could be obtained if tissue handling and staining protocols are implemented in laboratories in an attempt to decrease exogenous factors interfering in the staining with PG.

It also needs to be emphasised that Asimaki et al published their results based on immunofluorescence and confocal microscopy. They only gave a protocol for conversion to immunohistochemistry, not results from immunohistochemistry. It is likely that the variability and confounding factors which exist in immunohistochemistry as compared to immunofluorescence and confocal microscopy resulted in these discrepant results when it comes to demonstrate a loss of PG staining.

Findings from this study, as well as others, highlight both the technical difficulties in the pre-analytical stage of PG immunohistochemistry and the analytical stage of interpreting the results. A marked loss of PG immunohistochemical staining, when compared to N-Cadherin (staining intensity difference of -2 or -3), could be used to confirm a diagnosis of ARVC but not exclude it from the differential diagnosis. The authors therefore believe this stain should be used with caution in the routine work up of ARVC.

5. CONFLICTS OF INTERESTS / DISCLOUSURES

The authors have no conflicts of interest.

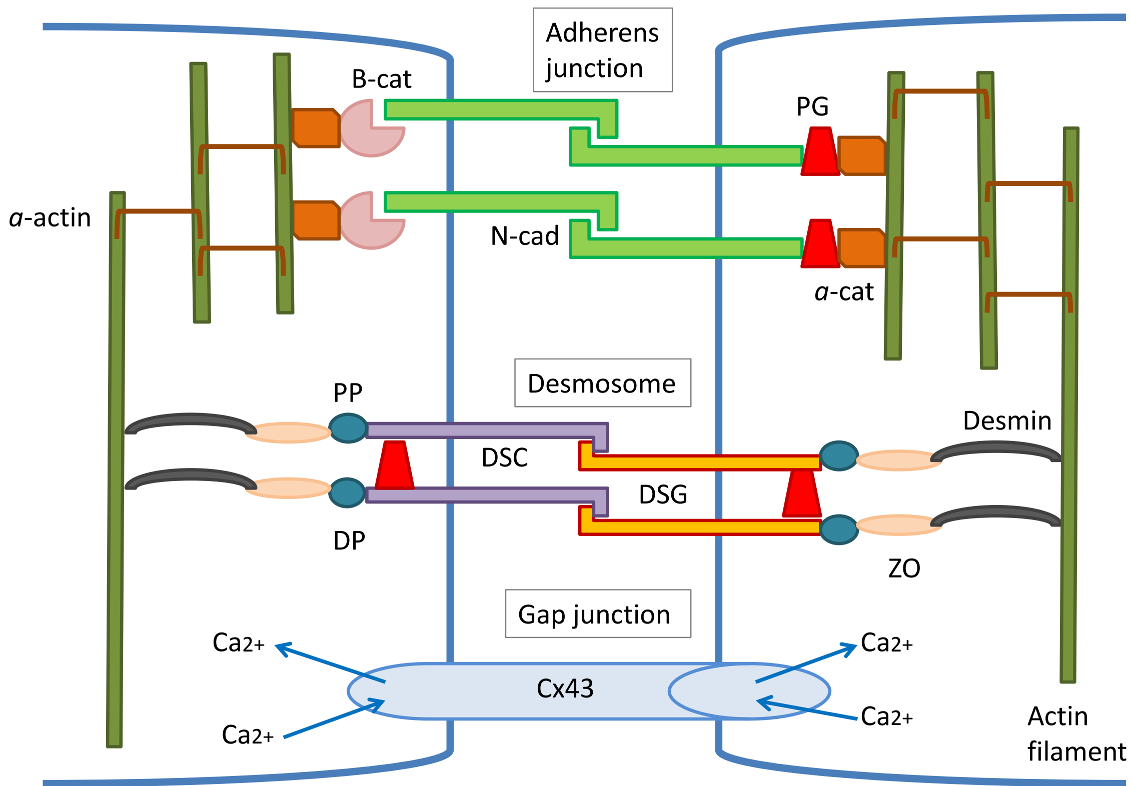


Figure 1: Schematic of the intercalated disc (adapted from Guo D; J Mol Cell Cardiol 2011 [55]). *a*-cat = alpha-catenin; B-cat = Beta – catenin; Cx43 = Conexin 43; DP= desmoplakin; DSC = Desmocollin; DSG = Desmoglein; N-cad = N-cadherin; PG = Plakoglobin; PP = Plakophilin.

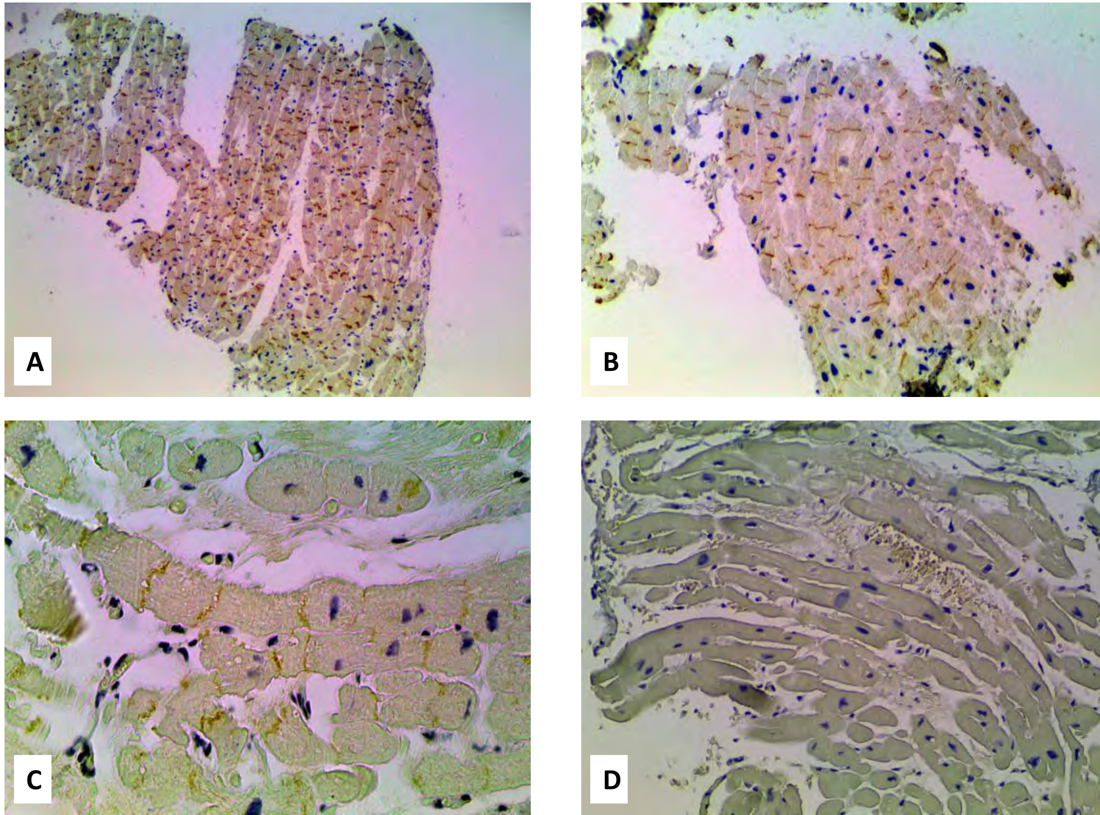


Figure 2: Staining intensity of placoglobin. A = score 3, 4x objective; B = score 2, 20x objective; C = score 1, 40x objective; D = score 0, no staining at any magnification.

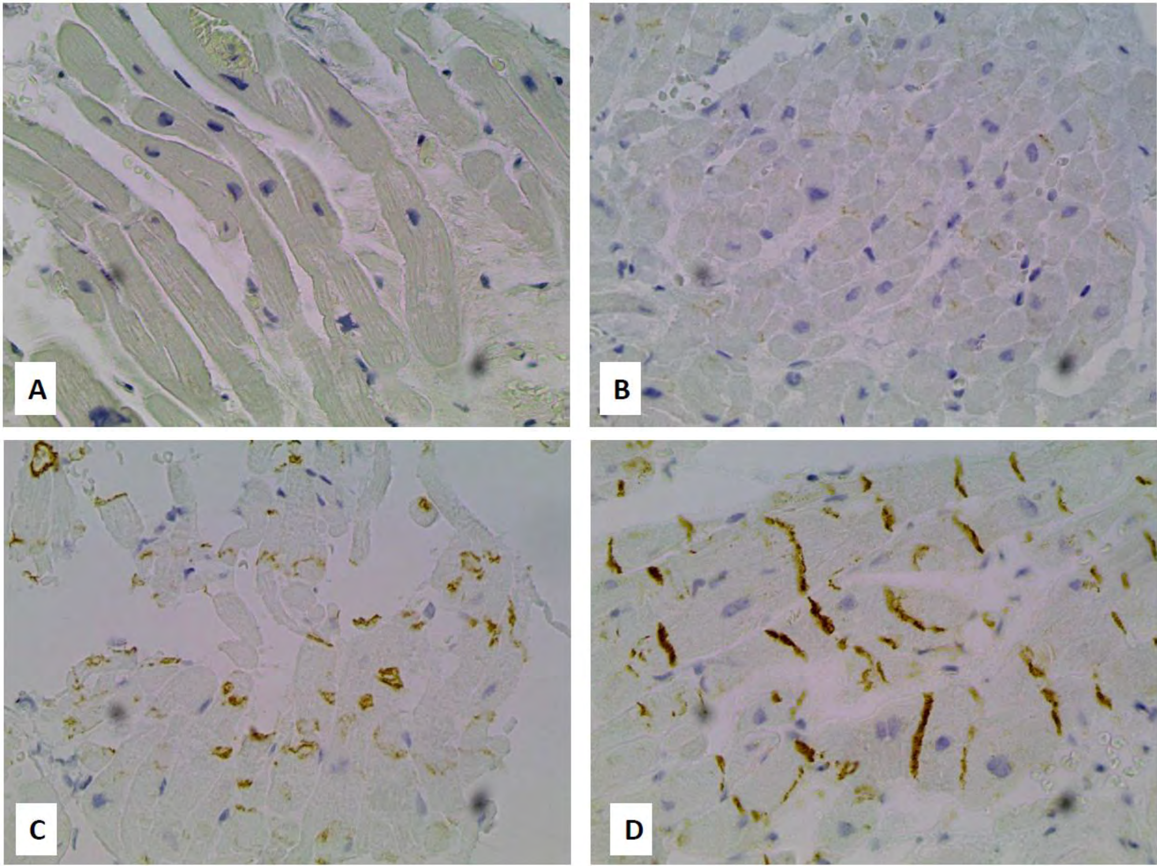


Figure 3: Staining intensity of Placoglobin at 40x objective
A = score 0; B = score 1; C = score 2; D = score 3

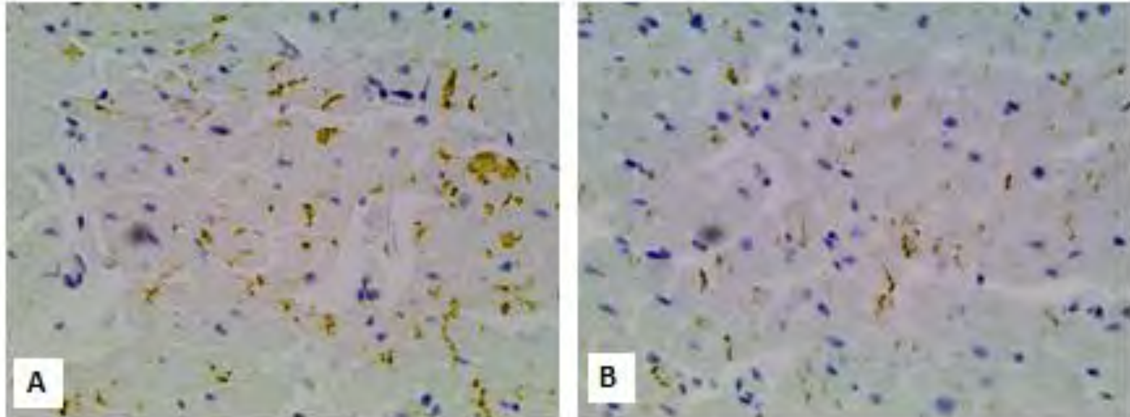


Figure 4: Staining intensity difference between Placoglobin and N-cadherin case number 40.
A = N-cadherin, score 3; B = Placoglobin, score 3.
Staining intensity difference = 0, therefore an immunohistochemical diagnosis of non ARVC.

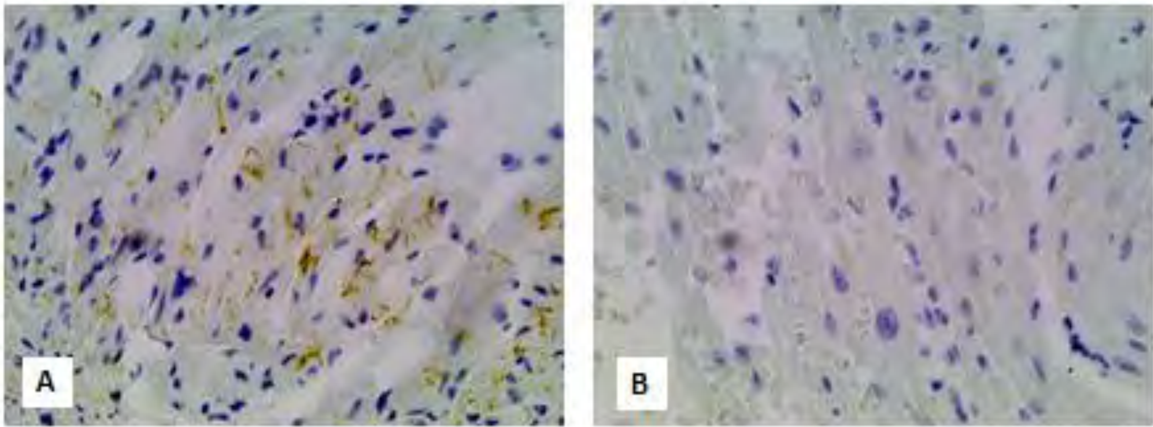


Figure 5: Staining intensity difference between Placoglobin and N-cadherin, case number 26
A = N-cadherin, score 3; B = Placoglobin, score 0.
Staining intensity difference = - 3, therefore an immunohistochemical diagnosis of ARVC.

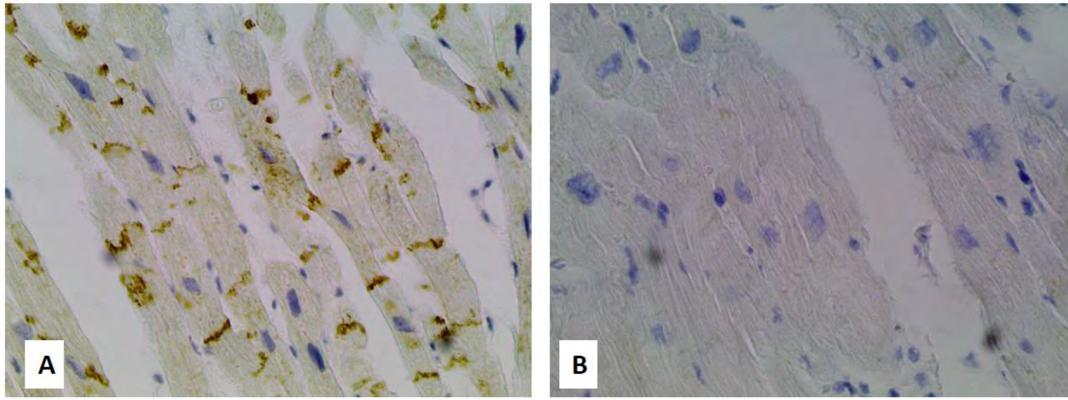


Figure 6: False positive diagnosis of ARVC, qualitative assessment, case number 8

A = N- cadherin; B = Placoglobin

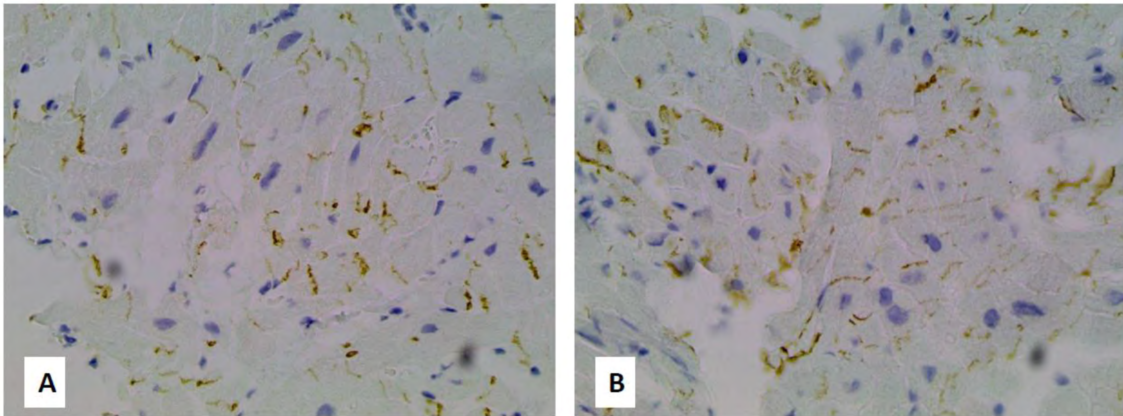


Figure 7: False negative diagnosis of ARVC, qualitative assessment, case number 15

A = N-cadherin; B = Placoglobin

LEGENDS FOR FIGURES

Figure 1: Schematic of the intercalated disc. *a*-cat = alpha-catenin; B-cat = Beta – catenin; Cx43 = Conexin 43; DP= desmoplakin; DSC = Desmocollin; DSG = Desmoglein; N-cad = N-cadherin; PG = Plakoglobin; PP = Plakophilin.

Figure 2: Staining intensity of placoglobin. A = score 3, 4x objective; B = score 2, 20x objective; C = score 1, 40x objective; D = score 0, no staining at any magnification.

Figure 3: Staining intensity of Placoglobin at 40x objective
A = score 0; B = score 1; C = score 2; D = score 3

Figure 4: Staining intensity difference between Placoglobin and N-cadherin case number 40.
A = N-cadherin, score 3; B = Placoglobin, score 3.
Staining intensity difference = 0, therefore an immunohistochemical diagnosis of non ARVC.

Figure 5: Staining intensity difference between Placoglobin and N-cadherin case number 26.
A = N-cadherin, score 3; B = Placoglobin, score 0.
Staining intensity difference = - 3, therefore an immunohistochemical diagnosis of ARVC.

Figure 6: False positive diagnosis of ARVC, qualitative assessment, case number 8

A = N- cadherin; B = Placoglobin

Figure 7: False negative diagnosis of ARVC, qualitative assessment, case number 15

A = N-cadherin; B = Placoglobin

Diagnostic category	Staining intensity difference (N-cadherin score subtracted from PG score)
ARVC	-2 to -3
Possible ARVC	-1
Not ARVC	0 to +2

Table 1: Semi-quantitative diagnostic categories

		N.J.M	H.C.W	Kappa
Semi-quantitative analysis				0.085
All diagnostic categories	Sensitivity	36.46% [95% CI: 14.00 % to 68.36 %]	23.08% [95% CI: 5.31 % to 53.80 %]	
	Specificity	39.22% [95% CI: 21.53 % to 59.42 %]	64.29% [95% CI: 44.07 % to 81.33 %]	
Excluding “Possible ARVC”	Sensitivity	27.27% [95% CI: 6.35 % to 60.92 %]	0% [95% CI: 0.00 % to 31.03 %]	
	Specificity	73.33% [95% CI: 44.91 % to 92.05 %]	85.71% [95% CI: 63.63 % to 96.78 %]	
Qualitative analysis				0.45
	Sensitivity	72.73% [95% CI: 39.08% to 93.65%]	63.64% [95% CI: 30.88% to 88.85%]	
	Specificity	32% [95% CI: 14.99% to 53.50%]	40% [95% CI: 21.16% to 61.32%]	

Table 2: Semi-quantitative and qualitative statistical analysis

6. REFERENCES

1. Thiene G, Corrado D, Basso C. Arrhythmogenic right ventricular cardiomyopathy/dysplasia. Orphanet J Rare Dis 2007;2:45

2. Tabib A, et al. Circumstances of death and gross and microscopic observations in a series of 200 cases of sudden death associated with arrhythmogenic right ventricular cardiomyopathy and/or dysplasia. Circulation 2003;108:3000-3005.

3. Markus F. Autopsy and endomyocardial biopsy findings.Arrythmogenic RV Cardiomyopathy/Dysplasia Recent Advances. In: Markus F (ed) Arrythmogenic RV Cardiomyopathy/Dysplasia Recent Advances. 1 ed. Springer-Verlag Itlia: Assago; 2007.

4. Basso C, Thiene G. Adipositas cordis, fatty infiltration of the right ventricle, and arrhythmogenic right ventricular cardiomyopathy. Just a matter of fat? Cardiovasc Pathol 2005;14:37-41.

5. Thiene G, et al. Right ventricular cardiomyopathy and sudden death in young people. N Engl J Med 1988;318:129-33.

6. Kumar V. The Heart. Robbins and Cotran Pathologic Basis of disease. In: Kumar V (ed) Robbins and Cotran Pathologic Basis of disease,7 ed. Elsevier Saunders; 2005, pg 555.

7. Saffitz JE, Hames KY, Kanno S. Remodeling of gap junctions in ischemic and nonischemic forms of heart disease. J Membr Biol 2007;218:65-71.

8. Thiene G. Arrhythmogenic right ventricular cardiomyopathy/dysplasia. Orphanet journal of rare diseases 2007;2:45.

9. Saffitz JE. Dependence of electrical coupling on mechanical coupling in cardiac myocytes: insights gained from cardiomyopathies caused by defects in cell-cell connections. Ann N Y Acad Sci 2005;1047:336-44.

10. Li J, et al. N-cadherin haploinsufficiency affects cardiac gap junctions and arrhythmic susceptibility. J Mol Cell Cardiol 2008;44:597-606.

11. Swope D, Li J, Radice GL. Beyond cell adhesion: the role of armadillo proteins in the heart. Cell Signal 2013;25:93-100.

12. Guo D, et al. Uncoupling between enhanced excitation-contraction coupling and the response to heart disease: lessons from the PI3Ky knockout murine model. J Mol Cell Cardiol 2011;50:606-612.

13. McKenna WJ. Diagnosis of arrythmogenic right ventricular dysplasia/cardiomyopathy. British heart journal 1994;71:215-218.

14. Awad MM, Calkins H, Judge DP. Mechanisms of disease: molecular genetics of arrhythmogenic right ventricular dysplasia/cardiomyopathy. Nat Clin Pract Cardiovasc Med 2008;5:258-67.

15. Swope D, Glenn JL, Radice L, Beyond cell adhesion: The role of armadillo proteins in the heart. Cellular Signalling 2013;25:93-100.

16. Asimaki A, et al. A new diagnostic test for arrhythmogenic right ventricular cardiomyopathy. N Engl J Med 2009;360:1075-84.

17. Munkholm J, et al. Usefulness of immunostaining for plakoglobin as a diagnostic marker of arrhythmogenic right ventricular cardiomyopathy. Am J Cardiol 2012;109:272-275.

18. Noorman M, et al. Reduced plakoglobin immunoreactivity in arrhythmogenic cardiomyopathy: methodological considerations. Cardiovasc Pathol 2013;22:314-318.

19. Asimaki A., et al. Altered desmosomal proteins in granulomatous myocarditis and potential pathogenic links to arrhythmogenic right ventricular cardiomyopathy. Circ Arrhythm Electrophysiol 2011;4:743-752.

20 Wolff A, et al. Recommendations for Human Epidermal Growth Factor Receptor 2 Testing in Breast Cancer: American Society of Clinical Oncology/College of American Pathologists Clinical Practice Guideline Update. J Clin Oncol 2013;31:3997-4013

21 Kuan, S. et al., Immunohistochemical detection of BRAF V600E mutant protein using the VE1 antibody in colorectal carcinoma is highly concordant with molecular testing but requires rigorous antibody optimization. Human Pathol. 45: p. 464-72

22 Landis, J., et al., The measurement of observer agreement for categorical data. Biometrics 33: p. 159-74

APPENDIX 1 – Modern Pathology Guide for Authors

Guide for Authors

Welcome to the electronic manuscript submission website for Modern Pathology. The instructions below are structured so you can quickly and easily answer the following questions:

Is my manuscript suitable for Modern Pathology? (Scope + Editorial Policy)

How do I format my manuscript for Modern Pathology? (Format of Papers)

How do I submit my manuscript to Modern Pathology? (Submission of Papers)

Modern Pathology is published monthly by Nature Publishing Group and is abstracted or indexed in:

Index Medicus/MEDLINE

Current Contents (Clinical Medicine, Life Sciences, Science Citation Index, SciSearch and Research Alert)

Excerpta Medica/EMBASE

Scope

Modern Pathology is an official Journal of the United States and Canadian Academy of Pathology and offers a recognized forum for publication of advances in the understanding of pathological processes. This journal serves as the practical complement to Laboratory Investigation, providing authoritative and clinically oriented articles selected to keep clinical pathologists informed regarding the latest developments in human diagnostic pathology. A thorough selection and review process ensures that the journal publishes the best papers from across the spectrum of applied pathology. In addition to original research, Modern Pathology also publishes letters to the Editor and, by specific invitation of the Editor, Editorials, Reviews, Short Courses and Special Topic articles. Authors wishing to submit manuscripts in the latter formats should do so only following approval by the Editor.

Editorial Policy

Editor-in-Chief:

Dr John. N. Eble, Van Nuys Medical, Science Building A128, 635 Barnhill Drive, Indianapolis, Indiana 46202

The Editor will review all material submitted for publication. Manuscripts that are considered to be of insufficient priority for publication will receive a prompt decision without further review. All other manuscripts are sent to two Editorial Board Members, and other expert consultants as needed. Peer reviewer identities are kept confidential. Author identities are not kept confidential.

All accepted manuscripts become the permanent property of the United States and Canadian Academy of Pathology, Inc., and may not be published elsewhere without written permission from the author(s) and the Academy.

Format of Papers

Preparation of manuscripts

Cover letter: Please include the name, institution and e-mail address of all contributing authors in your covering letter

Manuscript: All submissions should include the following:

Title Page. This should include: the full title of the paper, short, clear specific, and not more than 110 character; authors' names; and institutional affiliation.

Separate page giving name and address for page proofs, correspondence, and requests for reprints, and a running title of three to four words (no abbreviations, please).

Abstract. This should be submitted on a separate page and should describe in fewer than 300 words exactly what was done, the results obtained, and the conclusions drawn.

Text.This should appear with the following headings: “Introduction,” “Materials and Methods,” “Results,” “Discussion,” “Disclosure/Conflict of Interest,” “Acknowledgments” (optional), and “References.”

Charts, graphs, tables, and photographs. NOTE: all charts, graphs and tables will be printed in black and white. Color will only be available for the journal's online version only

Figure legends.

All text must be double-spaced with ample margins. All pages should be numbered consecutively starting with the title page as page 1. The author's name must appear in the upper right corner of each page. Footnotes are not accepted.

Non-Native Speakers of English: Authors who are not native speakers of English who submit manuscripts to international journals sometimes receive negative comments from referees or editors about the English-language usage in their manuscripts, and these problems can contribute to a decision to reject a paper. To help reduce the possibility of such problems, we strongly encourage authors to take at least one of the following steps:

Have your manuscript reviewed for clarity by a colleague whose native language is English.

Use one of the many English language editing services that are available, such as that offered by Nature Publishing Group Language Editing. An editor will improve the English to ensure that your meaning is clear and to identify problems that require your review.

American Journal Experts

Inter-Biotec

Inter-Biotec also provides a free online writing course to help biomedical scientists whose first language is not English to write and publish their papers in English-language journals.

Nature Publishing Group Language Editing

SPI Professional Editing Services

Write Science Right

Disclosure/Conflict of Interest: All authors are responsible for recognizing and disclosing any conflict of interest that could be perceived to bias their work in the acknowledgments, making known all financial support and any other personal connections. This includes, but is not limited to: funding, such as salaries, equipment, supplies, reimbursement for attending symposia, etc, from organizations that may gain or lose financially through the publication of the paper; personal financial interests, such as stocks and shares in companies that may gain or lose financially from publication, consultation fees or forms of remuneration from organizations that may gain or lose financially, or patent and patent applications whose value may be affected; and, employment, whether recent, present or anticipated, by an organization that may gain or lose from publication of the paper. If you have no conflict of interest to declare, please state so in this section.

Abbreviations, Nomenclature, and Symbols: These should conform as nearly as possible to those found in Scientific Style and Format: The CBE Manual for Author, Editors, and Publishers, 6th edition, Cambridge University Press, 1994. Please do not introduce an abbreviation in the title. Please do not introduce abbreviations in the abstract. Spell everything out completely. Modern Pathology discourages the use of ad hoc abbreviations. Modern Pathology does not include issue numbers in citations in reference lists. Modern Pathology does not italicize the abbreviations of journal titles in reference lists.

Gene Nomenclature Authors should use approved nomenclature for gene symbols and symbols rather than italicized full names (Ttn, not titin). Please consult the appropriate nomenclature databases for correct gene names and symbols. Approved human and mouse gene symbols are provided by HUGO Gene Nomenclature Committee (HGNC), e-mail: hgnc@genenames.org; see also http://www.genenames.org.

For proposed gene names that are not already approved, please submit the gene symbols to the appropriate nomenclature committees as soon as possible, as these must be deposited and approved before publication of an article.

Avoid listing multiple names of genes (or proteins) separated by a slash, as in 'Oct4/Pou5f' as this is ambiguous (it could mean a ratio, a complex, alternative names or different subunits). Use one name throughout and include the other at first mention: 'Oct4! (also known as Pou5f1)'

Types of Papers

Articles: Full Length Manuscripts should not exceed 20 standard letter-size pages including references. Manuscript length may influence decision to publish or not.

Letters to the Editor: The Editor welcomes brief letters commenting on articles appearing in recent issues. Those letters selected for publication will first be refereed to the senior author of the paper in question, whose response may also be published. The Editor reserves the right to reject any letter or to make any editorial changes he feels appropriate.

Invited Manuscripts: Editorial, Reviews, Short courses, and Special Topics are by invitation of the Editor. Authors who desire to submit such manuscripts should first receive approval by the Editor.

References

References are to be numbered in the order of citation within the article. Citations in the main text should appear as Arabic numerals in parentheses. References with less than six authors should list all names; for more than six authors, list the first three names followed by et al. Periodical abbreviations should follow those used by Index Medicus. Please note that issue number is not included in the citation. Individual references should be formatted in Vancouver style as follows:

Article

Smith AB, Jones CB. Alcoholic cirrhosis [abstract]. Lab Invest 1970;75:542-3.

Book

Smith JB, editor. Pathology of the lung. Vol 18. 3rd ed. John Green Co: Montreal; 1970. 179 pp.

Chapter

Brown AB, Green XY. Jejunal pathology, In: Black CD, White EF, (eds). Gastrointestinal pathology: an introduction. 2nd edn. Raven: New York; 1995. pp 465-469

Conference proceedings

Vivian VL, (ed). Pediatric pathology. Proceedings of the First International Conference on Pediatric Pathology; 15–19 Oct 1972; Chicago, USA. American Medical Association: Chicago; 1973.

References to articles in press must state name of journal and if possible, volume and year. References to unpublished material should be in parenthetical statements in the text. Authors must check references against original sources for accuracy; they must check every reference again in page proof.

Figures

Figures and images should be labeled sequentially, numbered and cited in the text. Figure legends should be printed and double-spaced on a separate sheet titled 'Titles and legends to figures' Figures should be referred to specifically in the text of the paper but should not be embedded within the text. The use of three-dimensional histograms is strongly discouraged when the addition of the third dimension gives no extra information. Graphs and charts should be prepared as black and white figures. If a table or figure has been published before, the authors must obtain written permission to reproduce the material in both print and electronic formats from the copyright owner and submit it with the manuscript. This follows for quotes, illustrations and other materials taken from previously published works not in the public domain. The original source should be cited in the figure caption or table footnote. Up to four (4) pages of color will be provided at no charge to the author at the discretion of the Editor-in-Chief. Any color pages beyond the four free pages will be charged $591.00 per color page

Figure legends

Legends must be submitted for all figures. They should be brief and specific, and should appear on a separate manuscript page after the Reference section. Use scale markers in the image for electron micrographs and indicate the type of stain used.

Artwork Guidelines

Detailed guidelines for submitting artwork can be found by downloading the guidelines PDF. Using the guidelines, please submit production quality artwork with your initial online submission. If you have followed the guidelines, we will not require the artwork to be resubmitted following the peer-review process, if your paper is accepted for publication.

Figures in Print

Authors are requested to follow our instructions on how to prepare their figures for more information see http://www.nature.com/aj/artworkguidelines.pdf. We request that at submission, ALL figures are of a high enough quality to be assessed in the peer review process. The file size of each submitted figure should not exceed 10 MB per figure; the aggregate file size for all figures submitted about not exceed a total of 50 MB.

Minimum Resolutions:

Halftone images 300 dpi (dots per inch)

Color images 300 dpi saved as CMYK

Images containing text 400 dpi

width=100>Line art 1000 dpi

Sizes:

Figure Width – single image 86 mm

(Should be able to fit into a single column of the printed journal)

Figure Width – multi-part image 178 mm

(Should be able to fit into a double column of the printed journal)

Text Size 8 point

(Should be readable after reduction – avoid large type or thick lines)

Line Width Between 0.5 and 1 point

Authors will be required to pay the cost of color illustrations published in print.

Color on the web

Authors who wish their articles to have FREE color figures on the web (only available in the HTML (full text) version of manuscripts) must supply separate files in the following format. These files should be submitted as supplementary information and authors are asked to mention that they would like color figures on the web in their submission letter.

For Single Images:

Width 500 pixels (authors should select "constrain proportions," or equivalent instructions, to allow the application to set the correct height automatically.)

Resolution 125 dpi (dots per inch)

Format JPEG for photographs

GIF for line drawings or charts

Filenaming Please save image with .jpg or .gif extension to ensure it can be read by all platforms and graphics packages.

For Multi-part Images:

Width 900 pixels (authors should select "constrain proportions," or equivalent instructions, to allow the application to set the correct height automatically.)

Resolution 125 dpi (dots per inch)

Format JPEG for photographs

GIF for line drawings or charts

Filenaming Please save image with .jpg or .gif extension to ensure it can be read by all platforms and graphics packages.

Authors may be asked to pay the full color fee for figures that are not submitted in the format described above.

Tables

These should be labeled sequentially as Table 1, Table 2, etc. Each table should be typed on a separate page, numbered and titled, and cited in the text. Reference to table footnotes should be made by means of Arabic numerals. Tables should not duplicate the content of the text. They should consist of at least two columns; columns should always have headings. Authors should ensure that the data in the tables are consistent with those cited in the relevant places in the text, totals add up correctly, and percentages have been calculated correctly. Unlike figures or images, tables may be embedded into the word processing software if necessary, or supplied as separate electronic files.

House Style

As the electronic submission will provide the basic material for typesetting, it is important that papers are prepared in the general editorial style of the journal.

See the artwork guidelines for information on labeling of figures

Do not make rules thinner than 1pt (0.36mm)

Color should be distinct when being used as an identifying tool

Color figure files should be set in CMYK format

Use Si units throughout

Spaces, not commas should be used to separate thousands

Abbreviations should be preceded by the words for which they stand in the first instance of use (abbreviations must not be introduced in the title or abstract)

Text should be double spacing with a wide margin

At first mention of a manufacturer, the town (state if USA) and country should be provided

Pages must be numbered. It is preferred that line numbering be used in the manuscript

File Formats:

File formats for manuscript files, figures and tables that are acceptable for our electronic manuscript submission process are given on the online forms. Further advice on file types is also available from the Tips webpage. Please follow our artwork guidelines for submitting figures, and use a common word-processing package (such as Microsoft Word*) for the text. Either embed tables converted into images at the end of your Word document, or as separate files in whichever program you used to generate them. If you submit raw data, this can be done in Excel or tab/comma delimited format.

Saving files with Microsoft Office

Microsoft Office saves files in an XML format by default (file extensions .docx, .pptx and xlsx). Files saved in this format or with a .doc extension are acceptable.

Select the Office Button in the upper left corner of the Word Window and choose "Save As"

Select "Word Document"

Enter a file name and select "Save"

These instructions also apply for the new versions of Excel and PowerPoint.

Equations in Word must be created using Equation Editor 3.0

Equations created using the new equation editor in Word 2007 and saved as a "Word 97-2003 Document" (.doc) are converted to graphics and can no longer be edited. To insert or change an equation with the previous equation editor:

Select "Object" on the "Text" section of the "Insert" tab

In the drop-down menu - select "Equation Editor 3.0"

Do not use the "Equation" button in the "Symbols" section of the "Insert" tab.

Supplementary information

Supplementary information is peer-reviewed material directly relevant to the conclusion of an article that cannot be included in the printed version owing to space or format constraints. It is posted on the journal's web site and linked to the article when the article is published and may consist of data files, graphics, movies or extensive tables.

The printed article must be complete and self-explanatory without the supplementary information. Supplementary information enhances a reader's understanding of the paper but is not essential to that understanding.

Supplementary information must be supplied to the editorial office in its final form for peer review. On acceptance the final version of the peer reviewed supplementary information should be submitted with the accepted paper.

To ensure that the contents of the supplementary information files can be viewed by the editor(s), referees and readers, please also submit a 'read-me' file containing brief instructions on how to use the file.

Supplying supplementary information files

Authors should ensure that the supplementary information is supplied in its FINAL format because it is not copy edited and will appear online exactly as originally submitted. Supplementary information cannot be altered, nor new supplementary information added, after the paper has been accepted for publication.

Please supply the supplementary information via the electronic manuscript submission and tracking system in an acceptable file format (see below). Authors should: include a text summary (no more than 50 words) to describe the contents of each file; identify the types of files (file formats) submitted and include the text 'Supplementary information is available at Modern Pathology's website' at the end of the article and before the references.

Accepted file formats

Quick Time files (.mov)

Graphical image files (.gif)

HTML files (.html)

MPEG movie files (.mpg)

JPEG image files (.jpg)

Sound files (.wav)

Plain ASCII text (.txt)

Acrobat files (.pdf)

MS Word documents (.doc)

Postscript files (.ps)

MS Excel spreadsheet documents (.xls)

PowerPoint files (.ppt)

We cannot accept TeX or LaTeX.

File sizes must be as small as possible so that they can be downloaded quickly. Images should not exceed 640 x 480 pixels (9 x 6.8 inches at 72 pixels per inch) but we would recommend 480 x 360 pixels as the maximum frame size for movies. We would also recommend a frame rate of 15 frames per second. If applicable to the presentation of the supplementary information, use a 256 color palette. Please consider the use of lower specification for all of these points if the supplementary information can still be represented clearly. Our recommended maximum data rate is 150 KB/s.

The number of files should be limited to eight, and the total file size should not exceed 8 MB. Individual files should not exceed 1 MB. Please seek advice from the editorial office before sending files larger than our maximum size to avoid delays in publication.

Further questions about the submission or preparation of supplementary information should be directed to the editorial office.

APPENDIX 2 – Raw data for subjective IHC assessment

Raw data for subjective IHC assessment 1 = ARVC, 2 = Non ARVC

Case number	HCW DIAGNOSIS	NJM Diagnosis	Clinical Diagnosis
1	1	1	DCM
2	2	1	HIV
3	1	1	HIV
4	1	1	HIV
5	2	2	ARVC
6	2	1	HIV
7	1	1	ARVC
8	1	1	HIV
9	1	2	Tx
10	1	2	Tx
11	1	1	DCM
12	1	1	ARVC
13	1	1	Tx
14	2	1	DCM
15	2	2	ARVC
16	2	1	HIV
17	1	1	ARVC
18	2	2	Tx
19	1	2	Tx
21	1	1	Tx
22	1	1	DCM
23	2	2	HIV
24	2	1	HIV
25	2	1	ARVC
26	1	1	ARVC
27	1	1	ARVC
28	1	1	ARVC
29	2	2	DCM
30	1	1	ARVC
31	2	2	HIV
32	1	1	Tx
33	1	1	Tx
36	1	1	Tx
39	2	2	ARVC
40	1	1	DCM
41	2	2	DCM

PART D: SUPPORTING DOCUMENTS

INTENTION TO SUBMIT

D1 (Amended) Approval of Study Proposal / Supervisor/s – updated on 13 Aug 2012

University of Cape Town

Faculty of Health Sciences

Form D1: Approval of Study Proposal (incorporating Supervisor Approval)

SUBMISSION OF STUDY PROPOSAL FOR A MASTER'S OR DOCTORAL DEGREE *AFTER ETHICAL APPROVAL*

PLEASE NOTE: This form must not be sent to Ethics

I would like to submit the attached proposal and supporting documentation for consideration by the Dissertations Committee (after Ethics approval).

Signature (Candidate): [signature] Date 24/07/2014

SURNAME OF CANDIDATE	MORSE	FIRST NAMES	NICOLE JOY
STUDENT NUMBER	MRSNIC013	PEOPLESOFT ID See student card	1 1 4 6 2 0 9
EMAIL ADDRESS	njmorse@gmail.com		
QUALIFICATIONS	MBChB, FC Path(SA) Anat		
TITLE OF PROPOSED PROJECT **(Proposal attached)**	AN IMMUNOHISTOCHEMICAL ASSESSMENT OF ENDOMYOCARDIAL BIOPSY SPECIMENS FROM THE SOUTH AFRICAN ARRHYTHMOGENIC RIGHT VENTRICULAR CARDIOMYOPATHY REGISTRY		
DEPARTMENT	DIVISION OF ANATOMICAL PATHOLOGY		
DEGREE NAME (e.g MSc (MED) IN HUMAN GENETICS)	MMed in Anatomical Pathology	DEGREE CODE	M
PROPOSAL APPROVED BY (Delete any one if not applicable) Human Ethics Committee, ERC No: ~~Animal Ethics Committee, ERC No:~~	If ethics approval not required please comment. **Please provide signature of Dept. Research Chair** 062/2010 [signature] 25th JULY 2014		
FINAL SUBMISSION OF STUDY PROPOSAL APPROVED BY SUPERVISOR	**Supervisor Name:** **Signature:** [signature]		
NAME(S) OF CO-SUPERVISOR(S)	1. B.M. Mayosi [signature] (Staff No: 01571250) 2. (Staff No:) 3. (Staff No:)		
FINAL SUBMISSION APPROVED BY HEAD OF DIVISION/OR DEPARTMENT	Name of **Head of Division**: **Signature** [signature]		

Please Note: Together with this form you must submit:

- **A copy of the Ethics approval letter (if relevant)**
- **A copy of the Study Proposal**

For office use:

Received by:	Name:	Date:	
Captured on PSoft / Database	Name:	Date:	
Entered in DC	Name:	DC no: PG-Med	Date:

The copyright of this thesis vests in the author. No quotation from it or information derived from it is to be published without full acknowledgement of the source. The thesis is to be used for private study or non-commercial research purposes only.

Published by the University of Cape Town (UCT) in terms of the non-exclusive license granted to UCT by the author.