Guidelines for consistent cardiovascular post-mortem examination, sampling and reporting of lesions in European zoo-housed great apes

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ABSTRACT

Inconsistencies and inadequacies in both the approach to post-mortem examination of the heart and the subsequent reporting of findings have been blamed for a lack of progression of understanding about great ape cardiovascular disease. In order to minimize and confront these issues in the United States, Association of Zoos and Aquariums-member zoos have necropsy data collated by the Great Ape Heart Project using a protocol developed by their pathology advisors. In Europe, however, there does not appear to be in existence any standardization or process for consistent cardiac post-mortem examination, data collection and/or relevant research. This article provides those readers working within/for European collections with a suggested protocol for the initial post-mortem inspection and sampling of the hearts of great apes. This article also outlines the detailed macroscopic and histopathological examination that is currently carried out at The University of Nottingham, UK, as part of an ongoing prospective, multi-centre study into great ape cardiovascular disease aetiopathogenesis. Finally, readers are offered guidance on the reporting and description specifically of idiopathic myocardial fibrosis (also referred to elsewhere as fibrosing cardiomyopathy). The standardized post-mortem examination of the great ape heart across European zoological collections will ensure a more uniform sampling procedure resulting in the availability of consistent good-quality data. It is hoped that this will, in turn, inform diagnostic pathology and research in this area, and enhance understanding about the aetiopathogenesis of great ape cardiovascular lesions.

Key-words: ape; cardiovascular disease; diagnosis; Hominidae; pathology; post-mortem examination; protocol.

INTRODUCTION

Cardiovascular disease has long been reported to be a significant cause of mortality among zoo-housed great apes (Meehan & Lowenstine, 1994; Gamble *et al.*, 2004; Lowenstine *et al.*, 2008; Nunamaker *et al.*, 2012; Strong, 2017; Strong *et al.*, 2017). Despite great efforts to date, however, there are significant gaps in our understanding about great ape heart disease epidemiology, pathogenesis, diagnosis and treatment. The critical need for the North American zoo community to investigate great ape cardiovascular disease was confronted by the establishment of the Great Ape Heart Project (GAHP). Based at Zoo Atlanta, GA, USA, the GAHP is a group of dedicated and coordinated subject-matter experts that provides a network of clinical, pathological and research strategies to aid in furthering understanding and reducing mortality associated with great ape heart disease, mostly across North America (Murphy *et al.*, 2018). The GAHP identified the following factors as being the main limitations to a progression in understanding in this field: (1) a lack of standardization with regards data collection across the taxa; (2) the absence of a comprehensive searchable clinical and pathological database; (3) a lack of dedicated research (GAHP, 2012). In

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2013, a review of current practices demonstrated that similar issues faced the European zoo community and also highlighted matters regarding the current approach to post-mortem investigation of great ape cardiovascular disease across Europe (V. J. Strong, unpubl. data).

In a review of data relating to the death of > 680 great apes that died in European zoological collections during the period 2004–2014, it was found that macroscopic and/or histopathological examination of the heart is not performed in every case (Strong, 2017). It was also shown that, when conducted, there can be issues with the approach and subsequent reporting of findings (Strong *et al.*, 2018). These factors limit not only the diagnostic power of the examinations performed but also the potential for meaningful comparison between the findings for multiple animals and, therefore, the scope for large scale multi-centre and longitudinal research studies. Additionally, the resulting paucity of comparable, good-quality biological samples available for research leads to competition for resources and further limits the potential for progressive research into great ape cardiovascular disease aetiology and pathogenesis.

In 2013, a taskforce was brought together with the aim of tackling these and other issues relevant to great ape heart disease research. The (European) Ape Heart Project, based at Twycross Zoo, UK, is a collaborative initiative endorsed by the European Association of Zoos and Aquaria (EAZA) Great Ape Taxon Advisory Group (GATAG) and is directed by one of their veterinary advisors. A collaborator of the USA-based GAHP, its role is to promote and drive the study of cardiovascular disease in great apes across European zoological collections.

One of the key outputs of this initiative to date has been a large-scale prospective study into great ape cardiovascular disease, carried out at The University of Nottingham, UK (Strong, 2017). This study is ongoing but to date 34 great ape hearts in total have undergone comprehensive macroscopic and histopathologic examination akin to that performed in the investigation of sudden cardiac death in people. The cardiovascular lesions identified have subsequently been described and characterized in detail (findings to be published separately), and compared with well-known pathologies that occur in people and other veterinary species.

At the time of commencement of this prospective pathology study, no guidelines for post-mortem examination of the great ape heart existed. A protocol based upon two papers on the cardiac autopsy published in the medical literature (Basso *et al.*, 2008; Sheppard, 2012), was therefore developed. Preliminary drafts of these protocols were tested in-house and amendments made accordingly. Further improvements were also made following consultation with a specialist medical cardiac pathologist and expert in (human) sudden cardiac death (M. Sheppard, pers. obs). Since this time, recommended guidelines for the cardiac necropsy examination and trimming for zoo vets and pathologists working in American zoological collections have been issued. The two (European

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and American) sets of protocols complement one another, their aims and objectives being well aligned and the data they generate being comparable.

This article provides those individuals working within/for European zoological collections with a suggested protocol for the initial inspection and sampling of the heart that should be carried out at the zoo as soon as possible after a great ape has died (Part 1). For the readers' interest and information, the article also provides an overview of the subsequent detailed macroscopic and histopathological examination that is currently carried out at The University of Nottingham as part of the ongoing study into great ape cardiovascular disease (Part 2). Finally, given that some zoological collections will still opt to submit the hearts to their own local pathologists, or even perform such diagnostics in house, the authors also provide readers with guidance about the reporting of great ape cardiovascular lesions (Part 3).

PART 1: INITIAL EXAMINATION, SAMPLING AND FIXATION

This article focuses specifically upon the examination of the cardiovascular system. However, the authors strongly advocate for a comprehensive post-mortem and systematic macroscopic and histopathologic examination of all organs. All cardiovascular lesions identified must be interpreted alongside diseases identified elsewhere in the body and in the context of the animal's history and recent health.

The initial inspection of the fresh heart should be performed as soon as possible after the animal's death by a suitably trained individual (ideally a pathologist or veterinarian), usually at the zoo or at a nearby facility. Given the risk of zoonotic infection when working with primates, personal protective equipment must be worn and appropriate measures must be taken when disposing of medical waste. The stages involved in initial post-mortem examination, sampling and fixation are hereby described.

1. Examination of the thorax
- The thorax should be opened and the lungs and pleural cavity inspected for the presence of any lesions or abnormal accumulations of fluid.
- A note should be made and a photograph taken of the heart's appearance *in situ*.

2. Examination of the pericardium
- The entire pericardial sac should be inspected and assessed for any lesions or the presence of any fluid.
- A section of the pericardium (including any areas with abnormal appearance) should be sampled and formalin fixed.

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3. Removal of the pluck

- The anatomy of the great vessels (especially for young animals) should be checked prior to sectioning.
- It is good practice to draw blood from the right atrium using a needle and syringe prior to opening. This blood can be cultured in cases of suspected septicaemia, or frozen whole for future diagnostic (including genetic tests) or research purposes. However, blood collection by this means might not be possible if the blood has clotted; in these instances the blood clot can be collected aseptically via a small incision in the atrial wall. If the animal is being euthanised, a perimortem blood sample is a suitable alternative.
- The pulmonary trunk should be cut transversely *c.* 3 cm above the pulmonary valve, the lumen opened and both branches of the pulmonary artery assessed for the presence of thrombi.
  - Post-mortem blood clots can be distinguished from thrombi by their lack of adherence to the endothelial tissue.

4. Removal of the heart from the pluck

- Lift the heart and separate the pulmonary veins from the left atrium.
- Separate the superior vena cava above and inferior vena cava below just above the diaphragm.
- All vessels should be transected 5 cm from the base of the heart and care taken to minimize iatrogenic damage to the heart structure.

5. Epicardial examination

- The heart should be rinsed in water and photographs taken of all sides (anterior, posterior, right lateral, left lateral, apex and base).
- The epicardial surface should be inspected for any lesions, thickening, changes in appearance, etc.
  - Some epicardial lesions (e.g. haemorrhage) can be associated with agonal change and death; they should nonetheless be described and their significance interpreted at a later date.

6. Opening of the heart

- A single transverse incision, perpendicular to the long axis of the heart and parallel to the coronary groove, should be made through the lower third of the apex to expose the chamber of both ventricles (Plate 1).
- Any clots should be removed and the heart rinsed in water.

- o Post-mortem sedimented blood clots can be distinguished from thrombi by their lack of adherence to the endothelial tissue.
- A photograph should be taken of the myocardial cross section and exposed ventricular chambers.

7. Weigh the heart

- The combined weight of both pieces of the heart (in grams) should be recorded.
  - o No more than the proximal 5 cm of the major vessels should be included.
- Special care must be taken to ensure that all clots have been removed prior to weighing.

8. Sampling of the apical myocardium

- A 1 cm x 1 cm x 0·5 cm portion of the sectioned piece of apex should be taken, frozen (at -80°C if possible or -20°C otherwise) and retained for future diagnostic (microbiological, viral) or research purposes.
- If RNAlater (stabilization solution: aqueous solution designed to maintain RNA integrity during storage of samples) is available, a second piece of myocardium *c.* 3 cm x 3 cm x 3 mm in size should be fully immersed in the fluid and also retained.

9. Fixation of the heart

- The remaining heart should be fully submerged in 10% neutrally buffered formalin ensuring all surfaces are covered and there is sufficient formalin around the heart (tissue/fluid ratio *c.* 1:10).
- Heavy hearts in particular (e.g. large adult males, gorillas) should be suspended in formalin (e.g. using string threaded through the great vessels) in order to prevent/minimize the heart becoming misshapen where it rests on the bottom of the container.
- The heart should be left to fix at room temperature for a minimum of 72 hours.

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A full post-mortem examination of the remaining carcass should also be carried out. As part of this, the aorta should be examined along its entire length and inspected for the presence of aneurysms, or atherosclerotic or any other lesions. Special attention should also be paid to the appearance of the lungs, liver, kidneys, eyes and brain, sections of which should also be formalin fixed and examined histologically. If an abnormal accumulation of fluid is noted at any stage, it should be quantified (in ml or weighed if clotted), characterized (colour, consistency, specific gravity) and, where possible, a sample stored. Photographs should be taken at all stages of the examination, particularly of any abnormalities identified. The animal's body weight (in kilograms), body condition (on a scale of 1 to 5) and crown to rump length (in centimetres) should also be documented.

The formalin-fixed heart should then be submitted for further, more detailed macroscopic and histopathologic examination by a suitably trained individual who is experienced at carrying out such examinations on great ape and/or human hearts.

PART 2: DETAILED MACROSCOPIC EXAMINATION AND TRIMMING
This section provides an overview of the protocol currently followed as part of the ongoing study into great ape cardiovascular disease at the University of Nottingham (Strong, 2017).

Upon arrival, samples are re-submerged/suspended in formalin to ensure complete fixation prior to further examination.

1. Assessment of the amount of adipose tissue
- It is normal for adipose tissue to be present within the atrioventricular groove, to extend along the course of coronary arteries and sometimes to cover the epicardium of the right ventricular anterior and lateral walls (rarely the posterior wall).
- In obese individuals, the adipose tissue can cover the entire epicardial surface, and spread into the myocardium along course of intra-myocardial vessels (especially the right ventricle and interatrial septum).

2. Subjective assessment of heart size and shape
- The heart is assessed for any overall increase or decrease in size, chamber dilation or wall thickening.
- The heart is carefully inspected for any gross anatomical abnormalities.

3. Epicardial examination
- The epicardium is inspected for the any lesions or colour changes.
  - Areas of discolouration are associated with, for example, haemorrhage or ischemic damage. However, epicardial mottling/discolouration is also a common artefactual finding caused by irregular formalin uptake and is poorly correlated with actual lesions; its presence should be noted but not be over-interpreted.
  - White patches on the epicardium (oligofocal, epicardial patches of minimal to mild collagen deposition with lymphocytic infiltrate) are a common finding, interpreted as incidental and likely age-related. In people these are called 'soldiers patches' and histologically they are composed of fibrosis and lymphocytes. Their cause is unknown, but theories include healed pericarditis and mechanical trauma. In people, they are thought to be of little clinical significance.

- Holes in the chamber walls are carefully inspected and their presence interpreted alongside other findings (e.g. assessed for free blood within the pericardial sac to distinguish ruptures and cardiac tamponade from iatrogenic post-mortem damage).

4. Inspection of coronary vasculature

- Transverse incisions are made at 3 mm intervals along the course of the main epicardial coronary arteries and their branches; the vessels are assessed for dilatation and/or atherosclerotic plaque formation.
- The origins of both coronary ostia/arteries are probed in their respective sinuses and checked for anomalous origins.

5. Heart weight

- All clots are removed before weighing.
- Those hearts that have significant parts of the cardiac structure missing are not weighed.

6. External measurements

- Heart length is measured from the coronary/AV-groove on the posterior aspect (Plate 2) to the tip of the apex.
- Heart circumference is measured at the level of coronary groove using string, which is marked and then measured against a ruler (Plate 2).
- All measurements are taken three times to ensure consistency and accuracy. Where three identical measurements cannot be obtained, a mean value is calculated.

7. Myocardial cross-section examination

- A fresh transverse incision is made across the lower third of the apex perpendicular to the long axis of the heart and ventricular chambers, and parallel to the coronary groove.
- The transverse myocardium is assessed for any evidence of mottling/colour change.
  - Colour changes can be caused by, for example, intra-myocardial haemorrhage, infarction or fibrosis. However, these changes can also be artefactual and associated with variation in formalin update and fixation.
    - Pink colouration of the mid-myocardium can be suggestive of inadequate formalin penetration and fixation.
    - The papillary muscles, sub-endocardial and/or subepicardial myocardium are often paler in colour than the mid-myocardium (Plate 3).

8. Wall thickness measurements

- The thickness of the following areas are measured at the mid-ventricular level:

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- o left ventricular free wall (LVFW);
- o interventricular septal wall (IVS);
- o right ventricular free wall (LVFW).
- Care is taken not to include the papillary muscles, epicardial fat or areas affected by focal lesions (e.g. thinning) in these measurements (Plate 3).

9. Internal chamber inspection

- The right atrium is opened (from the inferior vena cava to the right auricular appendage) and the right ventricle opened down the lateral aspect.
- The heart is assessed for an atrial septal defect or patent foramen ovale.
- The tricuspid valve circumference is measured just above the level of the cusps, using string, which is then measured against a ruler.
- The right ventricular outflow tract is opened by incising the anterior right ventricular wall along the paraconal interventricular groove (septum) and transecting the moderator band (trabecula septomarginalis).
- The pulmonary valve is measured at the commissures, using string.
- The left atrium and the left ventricle (to the apex) are opened down the lateral aspect.
- The mitral-valve circumference is measured (technique as for the tricuspid).
- The left ventricular-outflow tract is opened by cutting along the septum to the level of the aortic valve (note that the pulmonary artery will be transected by this action).
- The aortic valve is measured at the commissures, using string.
- The chambers and structures (papillary muscles, mitral valve, tricuspid valve, chordae tendineae) are inspected for abnormalities/lesions.

10. Examination of the aorta

- The aorta is assessed for any lesions, such as thickening, tears, dilatations, aneurysms, atherosclerosis, jet lesions.
- The existence and patency of orifices of left and right coronary artery are checked.

11. Examination of the pulmonary artery

- The pulmonary artery is assessed for any lesions, thrombi or an anomalous origin of a coronary artery from the sinuses.

12. Sampling for histopathology

- *Routine myocardial sections* The following samples (maximum 3 mm thickness) are taken for histopathology. Each is labelled with the animal's identification information and sample location. Sample locations are shown in Plate 4:

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- o   left ventricle: anterior wall (L1), posterior wall (L2), lateral free wall (L3);
- o   right ventricle: anterior wall (R1), posterior wall (R2), lateral free wall (R3);
- o   interventricular septum: anterior portion (S1), posterior portion (S2);
  - -   one side (in our cases, the left) should be immersed into marker dye to allow any pathologies that are restricted to the right/left side to be identified as such;
- o   right ventricular outflow tract;
- o   proximal aorta.

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Note: no sampling of atria or valves is carried out routinely, unless they appear macroscopically abnormal (e.g. infarction, tumour).

- *Conduction system* Given that relevant structures cannot be visualized grossly, sampling of the conduction system can be daunting. Nonetheless, taking samples of the sinoatrial node and atrioventricular node regions is good practice especially in cases where the death was sudden or unexplained, and so samples of the subepicardial sinoatrial node and subendocardial atrioventricular node are also taken as part of this protocol. Detailed guidance on how to sample these areas is provided in Sheppard (2012).

- *Additional myocardial sections* Further samples of any abnormalities or lesions identified are also taken.

13. Further examination of the myocardium

- Once all histopathology samples have been taken, transverse incisions (*c.* 1–1·5 cm intervals from apex to 2 cm beneath AV groove) are made to examine the remaining ventricular myocardium for infarcts/other lesions.

14. Histopathologic examination

- All tissue samples taken are processed, sectioned and stained.
- Haematoxylin and eosin stain is carried out as standard, additional specialist stains, such as Masson's trichrome (for connective tissue) or Congo red (for amyloid), are used only as indicated
- Each slide is examined in detail and histologic diagnoses generated.

PART 3: REPORTING OF FINDINGS

Usual principles for the reporting of pathological lesions should be followed, as for any organ or body system. However, several issues relating specifically to the reporting of cardiovascular

disease in great apes were identified by a recent study (Strong *et al.*, 2018). The following guidelines for the reporting of cardiovascular lesions were generated based upon these findings.

Nature of death

- Deaths should only be referred to as sudden if they were non-violent, unexpected and occurred within 24 hours of the animal last being seen in a stable, apparently healthy condition (Virmani *et al.*, 2001). A full history should be obtained, and clinical and keepers' records carefully reviewed to rule out any pre-existing clinical signs that might have indicated cardiac disease.

Terminology

- Terms such as cardiac or circulatory failure should be reserved for instances where disease of the heart was deemed to be the actual cause of death; it should not be used, for example, in cases where circulatory failure was the terminal result of an underlying condition, such as sepsis.
- Avoid use of ambiguous terms relating to size (e.g. enlarged, shrunken, etc.) without clarification using quantitative measurements (e.g. heart weight:body weight ratio; wall-thickness ratios) or histopathological features.
- Where possible, the same terminology used in other disciplines (e.g. domestic animal or human medicine) should be used. This allows comparison, is more meaningful and avoids ambiguity as to the meaning.

Distribution of lesions

- When describing the presence of any lesions, care should be taken to describe their location and distribution accurately; for example:
  - o chambers: left/right ventricles/atria, septum, etc;
  - o myocardial: subendocardial, subepicardial, mid-myocardial, random;
  - o inflammatory infiltrates: multifocal, disseminated, perivascular, lesion (e.g. fibrosis/infarct) associated.

Myocardial fibrosis

- When myocardial fibrosis is identified, its presence should be characterized/described in detail (e.g. severity, location, distribution). It must always be stated whether the increase of mature connective tissue or younger granulation tissue is replacement, perivascular or interstitial in type.

- o Interstitial fibrosis refers to an increase in collagen within the intermyocardial space and should be distinguished from replacement fibrosis, in which the connective tissue replaces normal myocardium. Perivascular fibrosis is an increase in collagen deposition expanding the adventitial and perivascular space.
- Detailed examination of numerous sections must be carried out to identify/rule out potential causes (e.g. infarction).
- Only if/when no cause has been identified following exhaustive examination, should the term idiopathic myocardial fibrosis (IMF) be used.
  - o The authors are of the opinion that the term fibrosing cardiomyopathy should be avoided in these instances for reasons outlined by Strong *et al.* (2018).

Other responses to injury

- Any other morphological changes/responses to injury that are observed (e.g. vacuolation, oedema, vascular changes, nuclear/myofibre hypertrophy) must also be described in detail.
- Any inflammatory infiltrates present should be described. The predominant cell type and distribution should both be stated.

Context

- Cardiovascular pathologies should always be reported/interpreted alongside other diagnosed pathologies in rest of the body. This allows for potential causative or contributing pathologies to be identified. Special attention should be paid to the lungs, brain, liver, kidneys and eyes. This allows for signs of heart failure to be identified and deaths associated with failure of cardiac output to be distinguished from those that result from dysrhythmias, as well as for vascular changes to be detected.
- If a genetic/familial condition is suspected [e.g. arrhythmogenic right ventricular cardiomyopathy (ARVC)] the zoological collection should be informed of the potential implications. They should also be advised to inform the veterinary advisor/species co-ordinator.

DISCUSSION

The examination of the great ape heart and subsequent reporting of findings is open to subjectivity and misinterpretation, especially in the hands of individuals for whom such examinations are not a regular occurrence. In addition, the level of examination required to gather diagnostic and research important information, as outlined in this article, is time consuming and labour intensive. Nonetheless, carrying out such a thorough and detailed examination is imperative for accurate diagnosis, and especially for future research and protection of the great ape population in zoos worldwide. For this reason, all European zoological collections and sanctuaries are encouraged to submit the hearts of any great ape that dies in their collection to the University of Nottingham

School of Veterinary Medicine and Science, for a free-of-charge, detailed macroscopic and histopathologic examination, providing further data for the ongoing (European) Ape Heart Project investigation into great ape cardiovascular disease.

Those working within the United States, AZA-member institutions follow a protocol that was developed by the GAHP pathology advisors. Whilst independent of one another, the two protocols were developed at a similar time and designed to satisfy similar goals. They are nonetheless complementary and the data they will generate comparable. The uptake and implementation of these protocols across Europe, the United States and beyond will generate a wealth of consistent data, information and samples to be collated. This will facilitate multi-centre comparative research studies into great ape cardiovascular disease aetiopathogenesis and diagnosis both now and in the future.

INFORMATION FOR THE READERS:
It should be noted that whilst the aim of the protocol is to promote complete and comprehensive examination of the entire great ape heart, its focus and bias is on the myocardium. This is because cardiomyopathies are the most prevalent cardiovascular disorders diagnosed among zoo-housed great apes (Strong *et al.*, 2018). A degree of discretion must be used on the part of the individual performing the examination. For instance, if there is clinical suspicion of a valvular disorder, the diagnostic approach might vary. Nonetheless, every effort should be made to ensure that most, if not all, of the same qualitative and quantitative information is collected where possible.

All data collected as part of the research study being carried out will be anonymized prior to publication to ensure that animals and submitting institutions cannot be identified. Access to samples/data held by the (European) Ape Heart Project will be controlled by the EAZA Great Ape Taxon Advisory Group and their veterinary advisors, and their use restricted to those research projects aimed at furthering understanding about great ape health and disease.

More information can be obtained from the project website:
https://twycrosszoo.org/conservation/research-at-twycross-zoo/current-research/ape-heart-project/

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CAPTIONS

Plate 1. The dashed line indicates the location of the transverse incision that should be made across the apex of the heart to expose the chambers of both ventricles during post-mortem examination of great apes. *School of Veterinary Medicine and Science, University of Nottingham.*

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Plate 2. Measurement of Chimpanzee *Pan troglodytes* **heart circumference is carried out using string. The star indicates the point in the coronary groove from which the heart length is measured (to the tip of the apex).** *School of Veterinary Medicine and Science, University of Nottingham.*

Plate 3. Myocardial cross section and ventricular chambers in a Chimpanzee *Pan troglodytes* **heart. The variation in colour associated with formalin penetration and fixation can be seen. The arrows demonstrate example measurements of wall thickness (note the exclusion of papillary muscles and epicardial fat from these measurements).** *School of Veterinary Medicine and Science, University of Nottingham.*

Plate 4. Location of the left (L1-L3), right (R-R3) ventricular and interventricular septal (L1, L2) wall sections to be taken for histopathology during post-mortem examination of great apes. *School of Veterinary Medicine and Science, University of Nottingham.*